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The effect of solute stress on the petite mutation in yeast

William 'Anitimoni Petelo
University of Wollongong

THE EFFECT OF SOLUTE STRESS ON THE *PETITE* MUTATION IN YEAST

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William 'Anitimoni PETELO, B.Sc. (Hons)

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

a_w	Water actitivity
BYM	Basal yeast medium
cAMP	Adenosine 3'5'-monophosphate
CFU	Colony forming units
log	Logarithm to base 10
LVC	Log viable count
MA	Malt extract agar
NADH	Nicotinamide adenine dinucleotide (reduced)
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised)
SDM	Standard deviation of the mean
TTC	2,3,5-triphenyl tetrazolium chloride

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ABSTRACT

An increased proportion of *petite* mutants was observed when mid-exponential cultures of *Saccharomyces cerevisiae* were transferred to a salt broth containing 10% NaCl as compared to a low spontaneous proportion that arose 'before transfer' (normal culture) to the salt broth. The proportion of mutants arising from salt exposure is a characteristic of individual yeast strains. This increase in proportion of mutants appeared to be due to the mutagenic action of the salt stress, rather then an enhanced survival of the pre-existing *petite* strains before salt stress. *Petite* mutants were not inherently more resistant to salt stress than the parent strains.

Changes in viability and proportion of *petite* mutants caused by a sudden transfer of mid-exponential cultures to salt broth were affected by: age of culture, temperature, and preconditioning (either in 2% NaCl or 45°C). Stationary phase cultures were resistant to transfer to salt broth as compared to mid-exponential cultures. Thus, the proportion of mutants was always low from stationary cultures, reflecting the spontaneous level before stress. The effects of temperature on salt stress-induced *petite* mutagenesis were also studied. High and low temperatures were used such as 20°C and 52°C. A temperature sensitive mutant, strain 182-6-3 (cdc 24), was used in experiments at 20°C and the wild type, Y41, and a previously considered to be trehalose negative mutant, 211-244-1A (*glc* 1), were used in experiments at 52°C. Results with cdc 24 showed an increase in *petite* mutagenesis when a mid-exponential culture was transferred to salt broth at 20°C. On the other hand, Y41 and *glc* 1 showed increases in *petite* mutagenesis when a mid-exponential culture was transferred to BYM at 52°C. However, when a Y41 mid-exponential culture was transferred to a salt broth (BYM containing 10% NaCl) at 52°C, the mutation rate was decreased and the apparent viability was increased compared to viability achieved from cells in BYM. This indicates that high NaCl concentration and high temperature oppose one another, thereby reducing the lethal effects on viability, and decreasing the mutation rate. Preconditioning of mid-exponential cultures either in 2% NaCl or 45°C also affects both the effect of transfer to salt broth (at 30°C) or BYM at 52°C. For example,

preincubation in 2% (m/v) NaCl suppressed the lethal effect of a sudden transfer to salt broth (10% m/v, NaCl at 30°C). Different effects were noted for the three yeast strains after such treatment. There was little protection for *glc* 1. The 2% NaCl pretreatment may be effective because it causes glycerol accumulation (Mackenzie et al. 1986) but the results obtained with *glc* 1 suggest that trehalose might also be involved in the stress protection. Pretreatment at 45°C protects against lethal effect of salt stress, but does not seem to suppress mutation. Both 2% NaCl and 45°C pre-conditioning suppress *petite* mutagenesis induced at 52°C.

Trehalose accumulated to a maximum of 18.7 and 4.1 mg. g^{-1}(dry mass of yeast) respectively within strains Y41 and *glc* 1. Trehalose may be a protector against heat and salt stress-induced *petite* mutagenesis.

CHAPTER ONE : LITERATURE SURVEY

1.1 THE *PETITE* MUTATION IN YEAST

1.1.1 Origin and initial characterization

The mitochondrial genetics of yeast began effectively with the discovery of *petite* mutants of *Saccharomyces cerevisiae* (Ephrussi et al. 1949a). Ephrussi discovered that a haploid/diploid culture of baker's yeast *S. cerevisiae* gave rise to two distinct colony sizes, *grande* (large) and *petite* (small) after plating on agar. Most of these colonies were *grande* with identical sizes, whereas, a minority (about 1-2 %) was comparatively small. This observation suggested that the yeast cell population was heterogeneous. Further experiments showed that the *grande* colonies always produced large (98%) and small (2%) colonies while the *petite* colonies yielded only small colonies when streaked on fresh agar plates (Ephrussi 1952). He also found that *petite* mutants could be induced in high proportion, even of up to 100 % by acriflavine or acridine (Ephrussi et al. 1949b). Table 1 is a brief outline of various agents or special treatments that induce the production of *petite* mutants in *S. cerevisiae* with high efficiency.

Ephrussi et al. (1949b) also found that when a *petite* mutant was crossed with a *grande* strain, the zygotes produced gave rise to *grande* diploids. When such diploids were sporulated, only a small proportion (less than or equal to 2.5%) of the ascospores produced gave rise to *petite* colonies (haploid spores). This incidence led to the first attempt to define the genetic characteristics of the *petite* mutant phenotype. Accordingly, two possible explanations were considered. The *petite* phenotype was caused by simultaneous mutation in a number of nuclear genes or loss from or damage to an extrachromosomal hereditary determinant (Ephrussi et al. 1949b). The latter explanation was more attractive to explain the high rates of spontaneous mutation. To test these hypotheses Ephrussi and his associates performed a series of crosses and back-crosses of the haploid *grande* isolates from the first cross with the original parent *petite*. Should nuclear mutation have been responsible, a gradual loss of the original *grande* parent genes by the serial back-crosses would have resulted in an increase in *petite* mutation progeny. This was not observed; hence, the second explanation was accepted and the mutation was considered to be an example of extrachromosomal inheritance.

Table 1.1 Agents / Inducers of *Petite* Mutation in *Saccharomyces cerevisiae*

Agent	Reference
Chemical Mutagens	
Sodium dodecyl suphate	Pinto da Costa & Bacila (1973)
N, N- (p-xylylidene) -bis-aminoguanidine.	Lachowicz et al. (1974)
N-methyl-N-nitroso-N-nitroguanidine.	Nordstrom (1967) Mayer & Legator (1970) Dawes & Carter (1974)
N-nitroso-N-methylurethane or nitrous acid and alkylating nitrosoamides	Schwaier et al. (1968)
Carcinogen (4 - nitroquinoline-1-oxide)	Nagai (1976)
Dithranol	Gillberg et al. (1967)]
Optical brighteners in washing powders, cosmetics , textiles and cigarette papers	Gillberg & Aman (1971)
Acriflavine (Euflavine)	Ephrussi et al. (1949a)
Euflavine component of acriflavine	Marcovich (1951) Mahler (1973)
Ethidium bromide	Slonimski et al. (1968)

Agent	Reference
INHIBTORS of mt-MACROMOLECULAR SYNTHESIS.	
mt DNA - Synthesis (weak inducers)	
Nalidixic acid	Gross &Smith (1972) Carnevali et al. (1976)
Phenethyl alcohol	Wilkie & Maroudas (1969)
mt RNA - Synthesis (efficient inducers)	
Rifampicin	Whittaker &Wallis (1971)
5 - Fluorouracil (5-FW)	Lacroute (1963), Oliver and Williamson (1976 a,b)
mt - protein synthesis	
Chloramphenicol	Weislogel & Butow (1970 ,1971)
Folic acid analogue (Methotrexate)	Wintersberger & Hirsch (1973 a,b)
Erythromycin	Williamson et al. (1971)

Agent	Reference
Temperature effects	
40°C	Yeas (1954)
54°C	Sherman (1959)
18°C	Weislogel & Butow (1970 &1971)
45 and 52°C	Schenberg-Frascino & Moustacchi (1972) Petelo (this thesis)
Nutritional and related influences	
Salt solution (KCl , NaCl, $CaCl_2$ or $SrCl_2$)	Yanagashima (1967)
NaCl	Petelo (1985)
Vitamin deficient medium (Asparagine as non C-source)	Nagai (1969)
2% Glycerol	Wallis et al. (1972) Wallis & Whittaker (1974)
Depletion of fatty acids	Marzuki et al. (1974)

Agent	Reference
Nutritional and related influences (continued)	
Divalent cation Manganese, copper, cobalt & nickel	Lindegren et al. (1958)
Cadmium	Nakamura (1961) Lindegren & Lindegren (1973)
Miscellaneous	
Ultraviolet light	Raut & Simpson (1955) Pittman (1959) Pittman et al. (1959) Maroudas &Wilkie (1968) Wilkie (1963) Allen &McQuillan (1969) Mayer & Legator (1970) Moustacci (1971) Johnson et al. (1973) Deutsch et al. (1974) Dujon et al. (1975)

In the first attempt to define the biochemical basis of the *petite* mutants, it was speculated that damage in the mitochondrial ATP-synthesizing system was responsible (Tavlitzki 1949; Slonimski 1949; Slonimski and Ephrussi 1949). Slonimski (1949) demonstrated that *petites* had low levels of oxygen uptake and were resistant to cyanide, an inhibitor of mitochondrial cytochrome oxidase. In addition, *petites* utilized glucose solely by fermentation whereas normal *grande* colonies could oxidize glucose completely by the mitochondrial respiratory chain system (Tavlitzki 1949). The mutants had an impaired respiratory electron transport system, lacking in cytochromes **a** and **b** but they did contain elevated levels of cytochrome **c** and respiratory enzymes such as succinate dehydrogenase and cytochrome oxidase (Slonimski and Ephrussi 1949).

Later work based on early studies by Ephrussi and Hottinguer (1950) suggested that *grande* yeasts contain an auto-reproducing extrachromosomal element essential for synthesis of some of the respiratory enzymes and that *petite* mutants arose when a component was not transferred in the bud. Genetic and biochemical studies supported this hypothesis at that time. Specifically, the lack of some cytoplasmic hereditary determinant was suggested by the fact that diploids resulted from original crosses of *petites* x *grande* inherited *grande* phenotype. However, some later studies on other *petites* , warranted a modification of the above view. Ephrussi et al. (1955) found that some *petite* strains,when crossed with a *grande* strain, produced some diploid *petite* colonies, the proportion of which was a characteristic of the particular *petite* strain. This phenomenon was called "suppressive" ; the percentage of which was termed "degree of suppressivity". Those diploids with zero suppressivity were called "neutral" and the others were called "suppressive". Neutral and suppressive *petites* are both respiratory deficient due to their impaired respiratory electron transport system. The third class, nuclear petites or *pet* mutants, is a result of a nuclear mutation which subsequently leads to respiratory deficiency (Chen et al. 1950). The subject matter of this review will be confined to neutral and suppressive *petites* .

Although many publications followed the discovery of petite mutants, progress was slow until it was reported that mitochondria in animal (Nass and Nass 1963) and yeast cells (Schatz et al. 1964; Tewari et al. 1965) contain DNA. It was realised that the mitochondrial (mt) DNA could be the extrachromosomal factor initially postulated by Ephrussi and later called " p factor " (Sherman 1964). The

molecular characterization of this DNA is now complete (see Section 1.1.5). In *petite* mutants, the mtDNA could be either lost (p^0 mutants) or grossly altered (p^- mutants). *Grandes* resulting from a p x wildtype cross are designated as p^+.

Suppressive *petites* (p^-) are heritable after crossing with the wild type yeasts (Ephrussi et al. 1955). Neutral petites, represented by p^0, do not inherit the p^0 phenotype/genotype in crossing with the *grande* yeasts (Ephrussi *et. al.* ,1955; Smith et al. 1969; Whittaker 1979; Wilkie 1982). When a haploid suppressive *petite* was crossed with haploid *grande*, the result was an equal proportion of *petite* diploid (p^-) and *grande* diploid (p^+) strains. p^+ is the only other type of cell in the zygote clones. Neutral *petites* (p^0) on the other hand completely lose mtDNA. A cross of both p^0 neutral *petite* haploid (p^0 x p^0) was found to be sterile; that is to say, no daughter cells were obtained (Wilkie 1982), an observation suggesting that eukaryotic cells might not grow and divide without mitochondria. *Petite* strains lacking mtDNA are now referred to as *rho* ^{0} rather than p^0 (see for example Dujon 1981). Although, both suppressive and neutral *petites* have different genotypes, they nevertheless produced similar phenotypes and could only be identified by physiological criteria.

Initial studies on *petite* mutants used colony size as a diagnostic criterion for the *petite* conditions but more precisely definitive techniques are now employed. Colonies can be grown on agar plates containing a non-fermentable substrate such as glycerol, lactate or ethanol as the major carbon source together with a small quantity of glucose (Mounolou 1967). Under these condition, *petite* colonies grow only until the glucose is exhausted. Since, they lack functional mitochondrial respiratory components, they cannot grow on non-fermentable carbon sources, whereas *grande* strains can. Another diagnosis is to overlay colonies with 2,3,5-triphenyltetrazolium chloride (TTC) which functions as a respiratory electron acceptor (Ogur et al. 1957). The *grande* wild type colonies reduce the dye to a red colour, whereas *petite* colonies remain white. A number of other techniques such as spectroscopic examination and use of non-fermentable carbon sources have been devised (Nagai et al. 1961), but are not commonly employed.

1.1.2 Induction of *petite* mutation

In relation to spontaneous *petite* mutation, and the use of acriflavine as a mutagen (see Table 1), a relatively wide range of physical, physiological and biochemical agents has been shown to be *petite* - mutagenic. These agents include temperature and nutritional effects, including a physicochemical stress such as that imposed by salt (10% m/v,NaCl; Petelo 1985), inhibitors of mitochondrial macromolecular synthesis (i.e. of mtDNA, mtRNA, and protein synthesis), ultraviolet-light, and many chemical mutagens (see Table 1). *Petite* mutants have been reported to be induced in both resting and growing cells. Acriflavine and ethidium bromide are both good examples of mutagens that could induce the mutation at any phase of growth. Both mutagens are inhibitors of both mtDNA and mtRNA synthesis, and are also DNA-intercalating agents (Waring 1965) in which role they modify the superhelical structure of DNA. In particular, ethidium bromide is the most effective *petite* inducer known and it can convert non-growing cells to a *petite* strain within a very short time (Slonimski, 1968).

High and low temperature effects and nutritional deficiencies have been reported to induce *petite* mutation (see Table 1). Schenberg-Frascino and Moustacchi (1972) found that a high proportion of mutants resulted when exponential cells were transferred from 28°C to 52°C, indicating that exponentially growing cells were much more sensitive than stationary resting cells to different effects of heating. It is now known that heat shock proteins (see Section 1.4) are associated with various effects of high temperatures in microorganisms. Prior to elucidation of the role of heat shock proteins, it was usually considered that protein denaturation was involved and nucleic acids were thought to be affected indirectly through the effect of heat on enzymes (Wood 1956). Additionally, heat shock delays cell division (Louderback et al. 1961), immediately disturb nuclear DNA synthesis in protozoa and mammalian cells (Evenson and Prescott 1970; Levine and Robbins 1970) and DNA extracted from heated cultures contains single stranded breaks (Bridges et al. 1969).

Nutritional deficiencies are also another factor that can induce *petite* mutation in *S. cerevisiae* . A number of bivalent cations such as manganese, copper, cobalt, and nickel (Lindegren et al. 1958) and cadmium (Nakamura 1961; Lindegren and Lindegren 1973) have been found to be *petite*- mutagenic.

According to Tzagaloff et al. (1975), manganese is a potent inducer of point mutations in mtDNA. This characteristic could, therefore, have affected the role of magnesium in mtDNA polymerase leading to faulty replication. Moreover, incubation of *S. cerevisiae* (var. *ellipisoideus*) cells without cell division in simple salt solutions (KCl, NaCl, $CaCl_2$ or $SrCl_2$) caused a gradual conversion of cells to *petite* mutants (Yanagashima 1967). It was suggested that cellular metabolism has a role in this induction, since low temperature or anaerobiosis prevents the mutation. Inclusion of glucose in the growth medium stimulated the induction of the mutation which was blocked by anaerobiosis. In contrast to Yanagashima's results, another study found that stationary phase cells of *S. cerevisiae* Y41 retained the same proportion of mutants as observed in the normal culture before being transferred to salt broth (10%NaCl), indicating resistance of resting cells to salt stress (Petelo 1985). Nevertheless, different strains of some species such as *S. cerevisiae* have been shown to respond differently to environmental stress such as water stress/solute stress. Starvation in 2% glycerol also generates *petite* mutations (Wallis et al. 1972; Wallis and Whittaker 1974). The rate of mutation is more pronounced when starved cells are harvested from the exponential rather than the stationary phase of the growth cycle. Depletion of fatty acids can cause *petite* mutation (Marzuki et al. 1974), principally of the suppressive type (p^-). This was suggested to be due to disruption of the mtDNA inner membrane complex involved in mitochondrial maintenance. The studies on starvation effects have suggested that a breakdown in the mechanism for maintenance of mtDNA caused by an imbalance in the nutrient source, may be responsible for the induction of the mutants (Whittaker 1979). Ultraviolet light and various other chemical mutagens have also been shown to induce mutation (see Table 1). It is now understood that usually the case treatments that induce *petites*, with the notable exception of a few potent mutagens, affect growing cells to a larger extent than non-growing ones.

Petite mutation can be blocked by various treatments and antagonists. Many studies have speculated about the site where the block of *petite* induction occurs (see the review article by Whittaker 1979). To explain the ethidium bromide-induced *petite* mutagenesis some inhibitors of the respiratory-chain and oxidative phosphorylation are postulated to antagonize the induction process by blocking the ATP-requiring nuclease step in the mutation process (Mahler and Perlman 1972; Meyer and Whittaker 1977). Other workers have suggested that the blocking effect of

anaerobiosis (Pinto et al. 1975) and of glucose repression of yeast mitochondria (Hollenberg and Borst 1971; Meyer and Whittaker 1977) indicates some other sites of protective action. Thus, one possibility is that a block on mtDNA transcription is caused by high glucose concentration which decreases the susceptibility of yeast mtDNA to nuclease attack (Whittaker 1979). Other antagonists such as rifampicin (Wallis and Whittaker 1974), daunomycin (Oliver and Williamson 1971) and also some types/forms of preconditioning (Section 1.4) have been found to block the induction of *petite* mutants.

1.1.3 Spontaneous mutation

As mentioned in Section 1.1.1, *petite* mutants arise spontaneously in yeast culture usually at a rate of 0.1 to 1.0 % per generation (Ephrussi et al. 1949a; Nagai et al. 1961). The frequency of *petite* mutation is a characteristic of individual strains (Whittaker 1979), and does not seem to result from variations in mtDNA levels amongst strains (Williamson 1970). James et al. (1975) found, following pedigree analysis, that new daughter cells are more likely to undergo spontaneous mutation than the parent cell. Two explanations for this observation were suggested; either there was a selective distribution of defective mtDNA to the bud at cytokinesis or the mother cells retained some factors that prevented the induction of defective mitochondria. Whittaker (1979) argued for the former explanation on the basis of some earlier results. In light of the finding that a yeast *grande* cell contains 50-100 mtDNA molecules (Williamson 1970 cited in Whittaker 1979), it is highly unlikely that spontaneous mutants arise by formation of daughters lacking any functional mitochondrial genetic determinants (Ephrussi and Hottinguer 1950 cited in Whittaker 1979). On the other hand, if the mtDNA was aggregated into a small number of clumps (Williamson and Funnel 1975), then it is possible, in conjunction with the smaller size of daughter cells, that some daughter cells could have missed obtaining one such clump of mtDNA molecules. This explanation, however, is unlikely in view of the finding (James et al. 1975) that most of the spontaneously arising mutants are p^- rather than p^o.

Some strains produce high proportions of "spontaneous" mutants. For instance, Oliver and Williamson (1976b) found that, of three strains of *S. cerevisiae*, two (temperature sensitive) had rates of spontaneous *petite* mutants at 25°C and 36°C of up to 5.9% and 5.6% respectively. In addition, a study using dithranol as *petite*

inducer (Gillberg et al. 1967) in a haploid strain of *S. cerevisiae* showed that the control culture (culture without dithranol) spontaneously produced *petite* mutants at a frequency of up to 16.0 % that arose spontaneously. Thus, the frequency of *petites* is a characteristic of an individual strain.

1.1.4 *Petite* - negative yeast

When a yeast strain does not give rise to viable extrachromosomal *petite* mutants, it is referred to as a "*petite* -negative yeast " (Bulder 1964). These have been reported for strains such as *Kluyveromyces fragilis* (Luha et al. 1974), *Kluyveromyces lactis* (Heritage and Whittaker 1977), and *Schizosaccharomyces pombe* (Heslot et al. 1970). On plating of a *petite*- negative yeast culture that was growing in the presence of a *petite* mutagen, it was observed that mutants were capable of producing only a few (0-10) divisions. These mutants produce microcolonies of less than 1000 cells (Heritage and Whittaker 1977) which are eventually autolyzed. Such microcolonies can be "rescued", however, by fusing with protoplasts from cells of respiratory-competent strains (Morgan et al. 1978).

1.1.5 Molecular basis of *petite* mutation

Studies on two genetically unrelated, acriflavine-induced, *petite* mutants were found to have an extensively altered base composition (4% GC) compared with mtDNA from the *grande* parental strain (18% GC) (Bernardi et al.1968; Mehrotra and Mahler 1968). Although the molecular basis of *petite* mutation was understood by 1979 (Bernardi 1979), its details including the phenomenon of suppressivity were only explained in the 1980s. Details about the mechanism can be found in reviews and articles by Bernardi (1979, 1982, 1983), Whittaker (1979) Dujon (1981), Baldacci and Bernardi (1982), Evans (1982), Wilkie (1982), Faugeron-Fonty et al. (1983), Mangin et al. (1983), de Zamaroczy et al. (1979, 1982, 1983, 1984), and Faugeron-Fonty et al. (1984). However, this review will briefly cover the aspects such as; organization of mtDNA (Section 1.1.5.1), deletion and tandem amplification phenomenon (Section 1.1.5.2), and some possible molecular mechanisms involved in the induction of *petites* by various mutagenic agents (Section 1.1.5.3).

1.1.5.1 Organization of mitochondrial DNA

1.1.5.1.1 Wild type mitochondrial DNA (p^+)

Most of the present knowledge on the wild-type genome was achieved by its comparisons with *petite* strains (p^-). *Saccharomyces cerevisiae,* like all other eukaryotic cells, has mitochondrial DNA which is chemically and physically different from DNA within the nucleus. It is now established that the mtDNA of wildtype yeasts is circular and contains about 75, 000 base pairs (bp); this corresponds to a length of about 25 μm or a molecular mass of 50 x 10^6 daltons (Hollenberg et al. 1969; Christiansen and Christiansen 1976; Sanders et al. 1977). The mitochondrial genome of *S. cerevisiae* is made up of long AT spacers (GC < 5 %) and about 200 short GC clusters (GC > 60%) (Bernardi 1983). The long AT spacers (50 % of genome) are formed by long stretches of short alternating and non-alternating AT : AT and A : T sequences (these sequences are characterized by a remarkable compositional homogeneity, and are highly responsible for the "anomalous" properties of yeast mitochondrial DNA such as buoyant density and melting point) with G : C occurring rarely. These sequences are internally repetitive and rich in palindromes (Bernardi and Bernardi 1980). The GC clusters, which have sequences that are often palindromic and homologous to each other, are contained within the AT spacers. AT spacers and GC clusters form the intergenic sequences of the genome and the intervening sequences such as *COB and oxi* 3 genes (see Sor and Fukuhara 1982). The mitochondrial genome of yeast contains (1) unique sequences, consisting of the genes coding for the RNAs (rRNA, tRNA, and mRNA) which are involved in the synthesis of polypeptide units for the respiratory enzyme complexes (other subunits are encoded in the nucleus); and (2) the repetitive interspersed sequences, mentioned above (AT spacers and the GC clusters) (see above).

1.1.5.1.2 *Petite* mitochondrial DNA

AT spacers and GC clusters of the wild-type mitochondrial DNA are a source of instability (Bernardi 1979) because they act as excision sequences (Zamaroczy et al. 1983) to cut a segment of the genome which is subsequently tandemly amplified to form the defective mitochondrial genome of a *petite.*

1.1.5.2 Deletion and tandem amplification phenomenon

Various studies (Bernardi et al. 1972; Piperno et al. 1972; Prunell and Bernardi 1977) found that 50% of the mitochondrial genome consists of AT spacers (GC <5%) which are homologously repetitive and long enough to allow site specific recombination. Current findings are consistent with a deletion mechanism (Bernardi et al. 1975) and accompanying tandem amplification of the excised genomic sequence (Locker et al. 1974; Bernardi et al. 1975), i.e. only a small proportion of the wildtype mtDNA was present in the *petite* genome (Figure 1). This excised segment is tandemly amplified to form the *petite* mitochondrial genome. The deletion and tandem amplification phenomenon describedhas been confirmed to explain the mechanism of *petite* mutation in the molecular level. Details can be found in reviews by Bernardi (1979, 1982,1983). This demonstration should be adequate for refuting some of the strange *ad hoc* hypotheses put forward by Slonimski (1968), Carnervali et al. (1969) and Borst and Kroon (1969) which have been alleged to explain the mechanism (Bernardi, 1982).

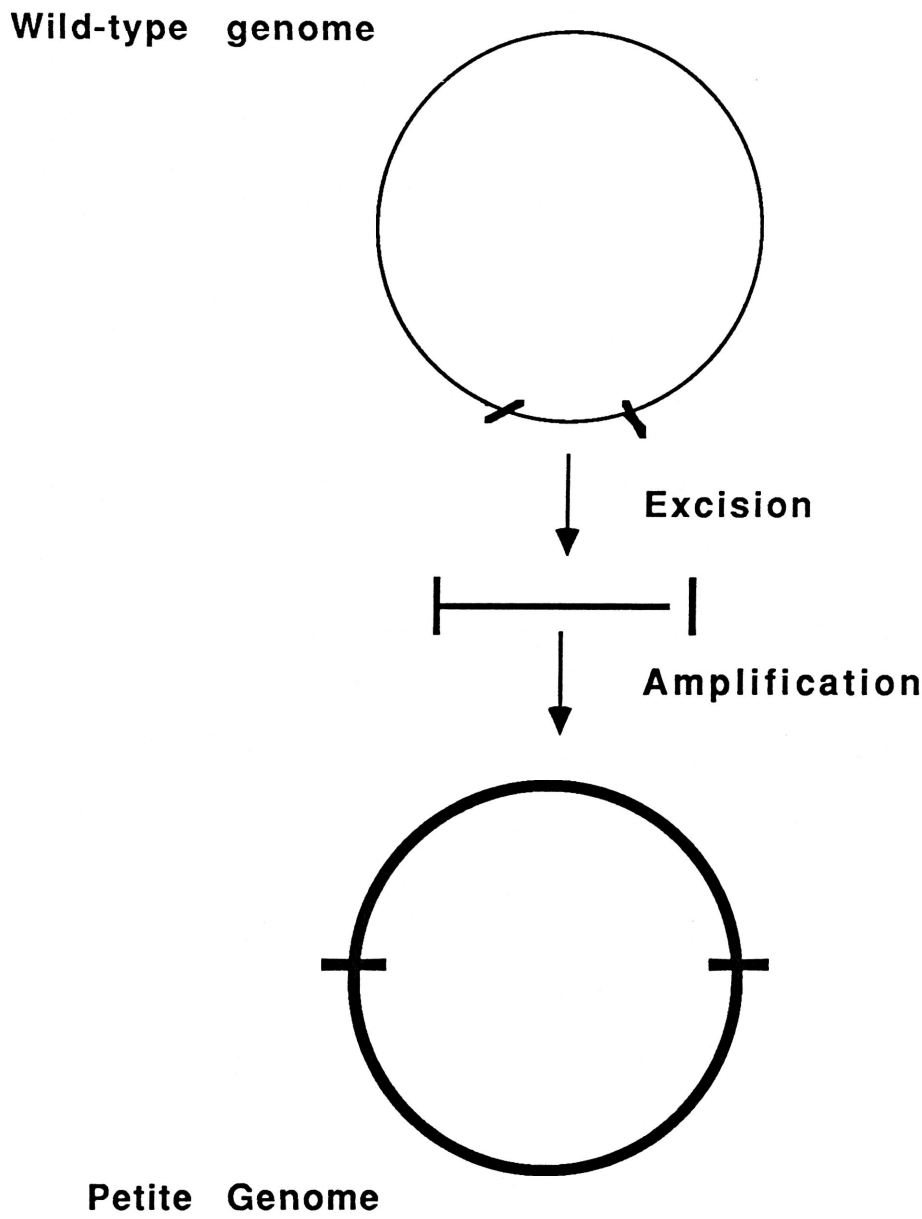


Figure 1. The diagram shows a scheme of the process leading to the formation of spontaneous *petite* genomes taken in its essentials from Bernardi (1979). A part of the mitochondrial genome unit from the parental yeast cells is excised and then amplified to form the mitochondrial genome unit of a *petite* mutant. The excised segment from the parental genome becomes the repeat unit of the *petite* genome. This may also undergo further deletions leading to secondary *petite* genomes with simpler repeat units.

1.1.5.3 Possible molecular mechanisms involved in the induction of *petites* by various mutagenic agents

Three mutagenic treatments are discussed in this section, first, because they are very mutagenic and, second, they have been extensively studied.

1.1.5.3.1 Ultraviolet light

Ultraviolet light (UV) efficiently induced *petite* mutation (Raut ans Simpson 1955) in haploid, diploid or tetraploid cells (Pittman 1959; Pittman et al. 1959). The UV treatment seems to make the cells unstable, since plating after UV treatment results in production of sectored (mixed *grande* and *petite*) colonies. The UV action spectrum for *petite* mutation has a maximum at 260nm which corresponds closely to that for the UV-induced killing of cells (Raut and Simpson 1955; Pittman et al. 1959; Maroudas and Wilkie 1968), implying nucleic acid as the likely primary target for UV-induced mutation (Whittaker 1979).

The effect of UV light varies with the state of repression of the mitochondrial system (Whittaker 1979). A study by Wilkie (1963) performed on both haploid and diploid strains found that aerobically grown cells showed sigmoid dose-response curves for UV light treatment in *petite* induction, suggesting a multiple-hit process for the mutation. When the cells were grown anaerobically, dose-responses curves were linear, indicating a single-hit process. A similar conclusion was reached by Allen and MacQuilan (1969), suggesting 20-UV mutable targets per cell in an aerobic, non-repressed culture and only 3 in anerobic cells. They assumed that the number of targets would correspond to the number of mtDNA molecules per cell. However, a later study found that there are only relatively small changes in mtDNA levels accompanying repression and derepression of the respiratory system (Williamson 1970). Whittaker (1979) suggested that the observed alterations in mutability may reflect differential activities of some processes subsequent to the primary mutagenic event but involved in the fixation of the mutation.

Growing cells seem to vary in susceptibility to *petite* induction by UV light treatment depending on their position in the cell cycle. They seem to be sensitive at the end of G_1 and the beginning of S phase which corresponds with the onset of budding (Chanet et al. 1973; Chanet and Heude 1974). Johnson et al. (1973) used

UV microbeam irradiation of budding cells. This was performed at the stage before the nucleus migrated into the bud to distinguish between the contribution of UV effects on the nucleus and the UV effects on the mitochondria to *petite* mutation. They found that the cells in which the nucleus was irradiated gave significant enhanced incidence of *petite* mutants, whereas cells in which the bud (still lacking a nucleus) was irradiated gave a proportion of *petite* insignificantly higher than the nonirradiated controls. Whittaker (1979) suggested that it seems that the nucleus is the primary target for UV induction of *petite* mutation, and that the subsequent loss of functional mtDNA is result of a breakdown of a nuclear-directed mechanism for maintenance of mtDNA. Moustacchi (1973) found that the induction of *petite* mutants by either UV or ethidium bromide was considerably less in recombination-deficient mutants of yeast. This suggests that nuclear gene products necessary for nuclear intragenic recombination events also contribute in steps of the metabolic chain leading to *petite* mutation (Whittaker 1979).

The lesion caused by UV light that leads to *petite* mutation is capable of undergoing both photorepair and dark repair. Photorepair has been shown for cells in which respiration is not repressed (Sarachek 1958). Photo-reversal of the mutation process was found to be under the control of a single nuclear gene showing simple dominance for the photoreactivable characteristic (Pittman et al. 1960). Photoreactivation capacity does not seem to be influenced by the particular point in the cell cycle at which UV followed by photoreactivating light is administered (Chanet and Heude 1974) or, to be affected by the growth phase of an irradiated culture (Heude and Moustacchi 1973). Photoreactivation is generally considered to be caused by splitting of pyrimidine dimers indicating that pyrimidine dimer formation is partially responsible for initiating the sequence of events leading to *petite* mutation (Moustacchi and Enteric 1970).

Studies on dark repair (excision-resynthesis) of the UV lesions leading to *petite* mutation initially indicated that at low doses of UV (less than or equal 1500 ergs/mm^2) for both haploid and diploid cells, a negative liquid holding (NLH) effect occurred (Moustacchi and Enteric 1970). That is, incubation of a UV-irradiated culture in saline for 4 days in the dark before being plated caused an increase in the level of mutants in the culture. Using a high UV dose, however, some recovery was observed during dark liquid holding. The growth phase of the cells is an important factor in whether dark repair is possible (Heude and Moustacchi 1973).

The NLH was observed when low-dose UV irradiation was applied to stationary phase culture. However, when exponential cultures were irradiated, dark liquid holding led to repair and reversal of the *petite* mutation (Heude and Moustacchi 1973).

1.1.5.3.2 Acriflavine (euflavine)

This is the first agent found to induce *petite* mutation in yeasts (Eprussi et al. 1949a). Acriflavine is a mixture of two acridines, proflavine and euflavine, where euflavine is considerably the more mutagenic of the two acridines (Marcovich 1951; Mahler 1973). Although proflavine and euflavine are different in mutagenicity, they are equally effective as inhibitors of mtDNA replication (Mattick and Nagley 1977). The two compounds are also equally effective as growth inhibitors for yeast. Mattick and Nagley (1977) suggests that both plasma membrane and mitochondrial membranes are permeable to proflavine.They suggest some permeability barrier to proflavine somewhere between these two membrane systems. They also observed that the majority of euflavine induced mutants are of the p^o type. As noted with other treatments, euflavine induced mutagenesis requires cell proliferation. Microdissection of budding cells grown under conditions of maximal euflavine has shown that a great majority of daughter cells (budded cells) gave rise to *petite* colonies, whereas mother cells remain *grande* (Marcovich 1951; Ephrussi and Hottinguer 1950). In addition, *grande* cells budding in the presence of euflavine gave rise to *grande* daughter cells while some daughter cells budding gave rise to *pette* colonies. It is difficult to envisage why *grande* and daughter cells behave differently since they seem to contain identical nuclear genetic information and should contain similar extranuclear cell contents. Perhaps the difference may lie in the metabolic process and cell sizes in the mother cells and newly formed daughter cells (Whittaker 1979).

Apart from acriflavine being a DNA-intercalating agent, this compound also inhibits mtDNA and mtRNA synthesis (Luha et al. 1971).

1.1.5.3.3 Ethidium bromide

Ethidium bromide (3,8-diamino-5-ethyl-6-phenylpheanthridium bromide) is at present, the most potent *petite* mutagen known. This chemical is a DNA-intercalating agent (Waring 1965) like euflavine, and it also modifies the superhelical structure of DNA. Unlike other mutagenic treatments which depends on cell proliferation in order for *petite* mutation to occur, ethidium bromide can convert a nongrowing yeast culture to *petite* mutants within a very short time (Slonimski et al. 1968). Studies by Slonimski and his colleagues showed a sigmoid dose-response curve as a consequence of the ethidium bromide *petite* induction. This suggested the involvement of more than one molecule of ethidium bromide in the induction process. In addition, these workers suggested that the alterations in the supercoiling of DNA following interaction with ethidium bromide initiate the breakdown of mtDNA. Like euflavine, ethidium bromide inhibits mtDNA replication (Goldring et al. 1970; Perlman and Mahler 1971) and mtRNA synthesis (Fukuhara and Kujawa 1970; Mahler and Davidowicz 1973). It binds strongly to mitochondrial membranes (Azzi and Sanatato 1971). In addition, ethdium bromide has also been found to stimulate a mitochondrial deoxyribonuclease in yeast (Paoletti et al. 1972) and act as uncoupler of oxidative phosphorylation (Miko and Chance 1975).

The molecular mechanism af ethidium bromide mutagenesis has received considerable attention. In a study where cycloheximide was added to a culture during ethidium bromide treatment, it was found through sucrose gradient centrifugation that the mDNA considerably reduced its size compared with untreated cells (Golring et al. 1970). Since cycloheximide blocks the synthesis of nuclear DNA (Grossman et al. 1969), the mtDNA synthesis could be studied against a low background level of nuclear DNA synthesis. The above study was confirmed by Perlman and Mahler (1971). These workers also demonstrated that during subsequent growth (of the already treated cells) in the absence of added ethidium bromide, a novel species of mtDNA appears. It is now apparent that this is the process of amplification of mtDNA fragments (see Section 1.1.5.2) which results in the presence in *petite* mutants of tandemly amplified sequences smaller than the whole *grande* mitochondrial genome.

In addition, ethdium bromide induced lesions which lead to *petite* mutation can be

considerably reduced by holding of the treated cells at 45°C in buffer prior to plating (Perlman and Mahler 1971). These workers suggested that the reversal is associated with heat-induced alteration in a membrane-mtDNA-ethidium bromide complex. This might also suggest a protective role of heat shock proteins induced at 45°C (see Section 1.4).

1.2 WATER STRESS IN MICROORGANISMS

Water is an indispensable component of life. All living things require water : from prokaryotes to eukaryotes, from micro- to macroorganisms. The availability of water varies from one environment to another. Microorganisms obtain inorganic salts and micronutrients from environments such as fresh-water (< 0.05 %, m/v, dissolved salts) and sea water (with total salinities 3.2-3.8% m/v, and above). Should the availability of water alter sufficiently to affect growth and reproduction of a microorganism, either by a decrease or increase, the microorganism would be subjected to a water or osmotic stress (Rose 1976). The parameter that is widely employed in measuring the availability of water in microbes is water activity (a_w) (see Section 1.2.1).

The changes of water availability that are responsible for water stress in microorganism are usually caused by changes in concentration of solutes. Thus a depletion of solute concentration amounts to a dilution stress in which an inflow of water to the microbial cell tends to cause swelling and sometimes the bursting of a cell. This can be prevented in many cases by a rigid cell wall which allows a positive hydrostatic (turgor) pressure to restrict the water entry and enlargement. Some wall-less fresh-water microbes can counterbalance the uptake of water by employing a contractile vacuole to remove excess water from the interior (Aaronson and Behrens 1974; Raven 1976).

1.2.1 Units of measurement

Water availability in microorganisms is usually described in thermodynamic terms such as water activity (a_w), osmotic pressure (π) or water potential (Ψ). The theory underlying the use of these units of measurement has been discussed in detail elsewhere (Nobel 1974,1983; Brown 1976; Dainty 1976; Zimmermann 1978;

Zimmermann and Steudle 1978; Gutknecht et al. 1978; Griffin 1981; Wyn Jones and Gorham 1983; Reed 1986). Nevertheless, a brief summary of their application is given below.

Water potential is a term derived from the chemical potential of water (see Dainty 1976) which is the partial molal free energy of water. If we consider a walled microbial cell then the water potential (Ψ) is equal to the sum of the hydrostatic (turgor) pressure (ψ_p) and osmotic potential (ψ_π); as given in equation 1.

$$\Psi = \psi_p + \psi_\pi \qquad (1)$$

At equilibrium, the water potential of the cell's interior is equal to that of the extracellular environment which, in turn, is affected by osmotic potential (ψ_π), and in special cases, by hydrostatic pressure and by an electrical term (see Griffin 1981; Nobel 1983). Matric potential (ψ_m) is implicated in biphasic interactions such as liquid-gas and liquid-solid, and is important in discontinuous environments such as soil. When the hydrostatic pressure and the electrical term are both ignored, the total water potential is represented by the following equations;

$$\Psi = \psi_p + \psi_\pi + \psi_m \qquad (2)$$

and, at equilibrium,

$$\psi_e = \psi_i = \psi_p + \psi_\pi + \psi_m \qquad (3)$$

where ψ_e and ψ_i respectively signify the extracellular and intracellular potentials.

Griffin (1981), in recognition of the role in timber of matric effects in solid environments, divided the dry environment into those where ψ_m is dominant (e.g. soil and stored food) and those where it is not (salt lakes, sugar syrups, brines). The validity of using matric potential contributions to the water potential arising from interface effects, however, has been questioned (see Nobel 1974; Passioura 1980). Passioura (1980, cited in Wyn Jones and Gorham 1983) had argued that an inconsistent definition of pressure caused misleading implications for water potential derived from interface effects. Accordingly, should the pressure be defined consistently as the hydrostatic pressure in the liquid phase, then any effect of solid surfaces can be included in P and π. Hence at equilibrium, we can derive the equation as;

$$\psi_e = \psi_i = \psi_P + \psi_\pi = P - \pi \qquad (4)$$

In addition, the intracellular matric potential is usually considered unimportant in microbes growing under optimal conditions (Adebayo et al. 1971; Griffin 1978) but might be significant for organisms subjected to a catastrophic decrease in the surrounding water potential (Schobert 1977). Water potential components are usually expressed in units of pressure (megapascals, 1MPa = 10 bar = 9.87 atm, at 25° C).

When Scott (1957) reviewed the water relations of food spoilage microorganisms, he recommended using the parameter water activity (a_w). Water activity has since been extensively used for the study of the effects of aqueous environments in microorganisms and it is a common practice for microbiologist to express water availability in this way.

Water activity bears a relation to Ψ in its natural logarithm although the ψ_p and ψ_m components in equation 2 are negligible in solutions. To arrive at the equation which defines water activity, let us consider that the proportion of water in a mixture

is at its mole fraction, $n_w/ (n_w + n_i)$, where n_w denotes the number of moles of water (the relevant substance) and n_i denotes the total number of moles of all other substances. Representing the mole fraction of water in a solution by N_w, Raoult's law states that in ideal solution;

$$N_w = n_w / n_w + n_i = P / P_0 \qquad (5)$$

where P and P_0 is the vapour pressure of solution and pure (water) solvent respectively. Hence, as the mole fraction of water decreases, the vapour pressure also decreases and as a direct consequence, the boiling point increases and the freezing point decreases. Thus, the mole fraction of water determines the colligative properties of a solution.

Equation 5 is only applicable to ideal solutions, but is valid over the entire concentration range at all temperatures between freezing and boiling point. However, ideal solutions are rarely encountered in practice although dilute solutions can provide a good working approximation of that state (Brown 1990). Concentrated solutions are different from ideal, thus an activity coefficient denoted by symbol γ is necessary to maintain a valid relation between the mole fraction and the vapour pressure. Hence, equation 5 is now modified to;

$$a_w = \gamma_w N_w = P / P_0 \qquad (6)$$

where $_wN_w$ is replaced by a new symbol, a_w, and called 'water activity' (see for example Robinson and Stokes 1965 and Brown 1990). In an ideal solution, the value of γ is 1.

There is an important relationship between water activity and the lowering of vapour pressure. In a closed container, the vapour pressure of a solution at equilibrium with the atmosphere is determined as a relative humidity (R.H.). That is to say, 100 x a_w is numerically equal to the percentage RH of an atmosphere with which it is in

equilibrium. Pure water is usually used as a reference with a_w = 1.00 and R.H. of 100%. All solutions have water activities less than 1. An example of various ranges of water activities which could be tolerated by some species of the yeast *Saccharomyces* is given in Table 1.2. As per Table 1.2, species of *Saccharomyces cerevisiae* can tolerate growing in a salt solution down to a minimum of a_w 0.93 whereas *Saccharomyces rouxii* tolerates growing even in a lower water activity with a minimum of a_w 0.75. In order for these yeasts to tolerate low a_w, they must accumulate compatible solutes.

The widespread use of water activity may be attributed to the simplicity with which it can be adjusted. In an ideal solution, water activity is independent of temperature whereas in a non-ideal solution, water activity is affected by temperature only to the degree that the activity coefficient (γ) is affected, which is largely negligible.

The parameter, water activity, has a lot of advantages to represent the status of aqueous systems not only because it is simple to deal both mathematically and experimentally, but also its independence of temperatures in real solutions. However, this parameter is unpredictable when applied to complex systems such as discontinuous environments like soil. In that case, we deal with water potential (Ψ) (see above).

Table 1.2 Approximate water activities for some species of *Saccharomyces* . From Pitt (1975) and Brown (1976).

Water activity (a_W)	Reference points	Foods	Saccharomyces Yeast
1.00	Blood		
	Sea water		
0.93			*S. cerevisiae* (in salt)
0.85	Salt lake		
0.75		Salami	*S. bailii* (in sugar)
			S. rouxii (in salt)
			S. rouxii (in sugar)
0.55	DNA disordered		

1.2.2 Osmoregulation

Microorganisms osmoregulate in order to survive changes in environmental water activity (see Section 1.2.3). The phenomenon, osmoregulation, is described elsewhere (see Brown 1976, 1978b, 1979; Brown and Edgley 1980; Griffin 1981; Noble 1974,1983; Wyn Jones and Gorham 1983; Reed 1984, 1986). Brown and Edgley (1980) defined osmoregulation as "the maintenance of approximately constant cell volume and turgor pressure in the face of changing water potential". Recently, Brown and associates (Brown et al. 1986) used another working definition of osmoregulation as "the maintenance of turgor pressure and/ or cell volume within limits necessary for growth and multiplication of an organism".

Osmoregulation can be studied at two distinct levels (Brown et al. 1986). One is biophysical, in which the physical parameters such as membrane potential and turgor pressure can be directly measured. The other is biochemical, in which the biochemistry and diverse aspects of the cell physiology of osmoregulation are analyzed (see Section 1.2.3).

When an organism is subjected to a change in environmental water activity, the following sequence of events occur. These can be characterized by three phases of adaptation (see Brown 1976, 1978a, 1979). Phase 1 is characterized by a rapidly thermodynamic adjustment of the organism to the new a_w; the water is osmotically driven inward (dilution stress or upshock; in system with increased a_w) or outward (solute stress or downshock; in case of low a_w) the cell. This movement of water (water flux) may be associated with cell shrinkage (downshock) or swelling (upshock) in wall-less microbes such as algae *Dunaliella salina* (Trezzi et al. 1965) and *Poterioochromonas malhamensis* (Kauss 1977). When the organism survives phase 1, it proceeds to phase 2. If it survives, it should return to approximately its original volume (wall-less organism) or turgor (walled organism). In some cases, complete recovery of original volume or turgor to original 'before' stress levels does not occur. Nevertheless, some organisms are still able to enter phase 3, which is marked by commencement of growth. Incomplete recovery of original turgor pressure following changes in environmental water activity has been observed in some species of microalgae (Kirst and Bisson 1979; Dickson et al. 1980; Reed et al. 1980), however, this does not seem to be a characterisitic of all microalgae (see for

example Bisson and Gutknecht 1977). Similarly, incomplete original cell volume recovery has been found in wall-less alga *Teraselmis subcordiformis* (Kirst 1977) whereas another genus of wall-less alga, *Poteriochromonas* , does recover original volume after a change in environmental a_w (Kauss 1977). In addition, recovery of original turgor or volume to a point where growth is possible could be achieved in phase 2 either by absorbing water or releasing water. These changes in cellular water content can be accomplished by adjusting solute content, which may be associated with metabolic conversion inside the cell or solute transport, or both. Once the solute content changes, the water fluxes maintain the thermodynamic parity between cellular and environmental water potential. A microorganism reaches phase 3 when growth starts at the new environmental water activity.

In phase 3, the organism is fully adapted to its new conditions bearing different phenotypes from that of the previous conditions (Brown 1976, 1979). Brown (1979) argued that it is presumably a matter of semantics whether or not this phase represents a stress because it does not imply any abnormal osmotic stresses. He referred to extreme environments such as saturated salt lakes and concentrated sugar solutions, which are ultimately inhibitory and lethal to most of the microorganisms. For this reason, those microorganisms which survive are highly selected. Halophils and halotolerant organisms (see Section 1.3) and a group of xerotolerant yeasts and moulds (*Saccharomyces rouxii*) are organisms that are successful in environment of low water activities.

In this thesis, 'osmoregulation' will be used according to the definition by Brown et al. (1986), because it is more flexible than the earlier definition of Brown and Edgley (1980) to deal with. The suitability of the term "osmoregulation" to explain the maintenance of approximately constant turgor and/ or volume (see above) has been questioned by Cram (1976) and Reed (1984, 1986). Turgor regulation (in walled cells) and volume regulation (in wall-less cells) were suggested as being informative terms, since the interrelation between turgor and volume (Bisson and Gutknecht 1980) indicates that it is hard to distinguish between turgor and volume regulation. Some other terms are suggested by Reed (1984, 1986).

1.2.3 Compatible solutes

The concept of compatible solute was first proposed by Brown and associates to explain accumulation of inorganic salt or ion by halophilic bacteria (Aitken and Brown, 1972) and one or a variety of polyhydric alcohols by sugar tolerant yeasts (Brown and Simpson 1972). Hence, they suggested that microorganisms in general respond to diminished water activities by accumulating at least one intracellular compatible solute, i.e. to function as an osmoregulator and/ or protector of enzymes against both inactivation and inhibition (Brown 1978a). It is now known that there are protective substances that fit the earlier definition (Brown 1978a) but, which are not osmoregulatory. Therefore, in recent years, the meaning of the term 'compatible solute' has been modified by the researcher who originally proposed the term and concept to clearly distinguished between compatible solutes and osmoregulatory solutes (e.g. Brown et al. 1986). The essential function of compatible solutes is that they protect against potentially lethal effects of a sudden solute stress, but do not necessarily accumulate in response to that stress. Thus, in order for a microorganism to tolerate lower levels of a_w, it must possess compatible solutes. In order to adapt from one water activity to another, it must osmoregulate. This is done by regulating the content of one or more osmoregulatory solutes.

At high water activity, the concentration of an osmoregulatory solute is sufficiently low, and can enable intermediary metabolites or inorganic ions that commonly accumulate to perform this role without causing any inhibition or toxicity (Brown et al. 1986). Hence, in a dilute solution, a relatively wide range of osmoregulatory solutes is encountered. In the case of low water activity, however, the osmoregulatory solutes are often restricted. Under such condition, an osmoregulatory solute may attain a high intracellular concentration (several molal), so toxicity is a potential problem. Thus, if an organism adapts to this environment, an osmoregulatory solute must be 'compatible' with the entire spectrum of essential cellular functions. In eukaryotic microorganisms, the only osmoregulatory solute which has been identified to perform such a function at low levels of water activity is glycerol (Brown 1978). Examples of eukaryotes that osmoregulate using glycerol are; the non-tolerant yeast *Saccharomyces cerevisiae* , the xerotolerant yeast *S. rouxii* , the salt-tolerant yeast *Debaromyces hansenii* , and the salt-tolerant alga *Dunaliella* (see Brown 1978, Adler and Gustafson 1980, Brown and Borowitzka 1979).

Accumulation of compatible solutes in response to a decrease in environmental water activity in various microorganisms has been tabulated by Borowitzka (1981) and Yancey et al. (1982). Four classes of compatible solutes have been found in microorganisms such as ; polyhydric alcohols, carbohydrate derivatives, K^+ (KCl) and free amino acids and their derivatives. The cellulaar concentration of such solutes has been observed to be high in xerotolerant and xerophilic microorganisms growing at extremely low water activities. For example, in *Halobacterium salinarium* growing in 4.0M salt, K^+ ions accumulated to about 4.6 molal (Christian and Waltho 1962) and similar or higher concentrations of K^+ have been reported in other halophilic bacteria.

In order to explain the capability of a microorganism to grow with high solute concentrations inside and outside the cell, Brown (1976) suggested two possible mechanisms. An organism using mechanism 1 has proteins that are characteristically different from those of other microorganisms and, thus, able to function inherently better under low water activities. Those organisms employing mechanism 2 modify their intracellular conditions in order to diminish the inhibitory effect of the environment on enzyme activity and cellular function. If distinctive metabolic pathways are used by a microorganism at low water activities, further subdivision is possible into those that contain enzymes which function intrinsically better at low water activities (mechanism 1A) or those that produce an end product that modifies intracellular conditions so as to diminish the environmental inhibition caused by low water activities (mechanism 2A). The salt requirement of halophilic bacteria can be largely explained in terms of mechanism 1. However, because the intracellular environment of these bacteria is modified they also employ mechanism 2. The metabolic pecularities concerned with salt requirement of these bacteria have been discussed by Brown (1983) and Kushner (1978,1985, 1986).

The water relations of xerotolerant and xerophilic eukaryotes can be largely explained by mechanism 2. All osmoregulatory solutes that are accumulated in these conditions (high solute concentrations) have been called 'compatible solutes' (Brown and Simpson 1972, Aitken and Brown 1972) because at high concentrations they allow enzymes to function effectively. Moreover, additional classes of compatible solutes in microorganisms such as amino acids and betaines are described elsewhere (Borowitzka 1981; Yancey et al. 1982; Truper and Galinzki

1986) (see also Section 1.3.2).

To a large extent, organic solutes rather than inorganic salt are used under extreme condition in virtually all organisms (eukaryotes and the halobacteria). The effectiveness of organic solutes as compatible solutes on extreme water stress may be attributed to their low molecular weight and highly water soluble molecules which are usually uncharged at physiological pH values. For instance, among amino acids, alanine is more soluble than glycine with a respective water solubility value of 3.3 mol/kg and 12.6 mol/kg (Truper and Galinski 1986). In addition, N-methylated amino acids, the betaines, are highly soluble in water and also found to be the most effective compatible solutes in a number of prokaryotes. Besides those solubility differences, Schobert (1977) claimed that proline and glycine betaine are both neutral at physiological pH, containing no aliphatic chain, and having little affinity to macromolecules or effect on water structure. However, Truper and Galinski (1986) pointed out that the only problem for the suitability of amino acids as compatible solute is due to the fact that only a few of them are present in their zwitterionic form at physiological pH.

1.3 RESPONSE OF YEASTS TO SOLUTE STRESS

'Stress' is defined in the Concise Oxford Dictionary as, "an effort or demand upon physical energy". Some stresses are temporary (reversible change), whereas others are permanent (irreversible change). Temporary and permanent stresses are often referred to as elastic and plastic (inelastic) stresses respectively. When an organism undergoes plastic stress, a permanent injury or mutation often results in emergence of a new strain or species. This thesis is concerned with a form of plastic change called *petite* mutation which is caused by solute stress (salt stress) (see Section 1.1). These mutagenic events occur when mid-exponential phase cultures of *S. cerevisiae* are transferred to salt broth (BYM containing 10% m/v, NaCl).

A great number of yeast species have been isolated from various natural and artificial low water activity habitats. Species from various genera such as *Candida, Debaryomyces, Hansenula, Pichla, Torulopsis* and *Saccharomyces* predominate in these habitats. Their distribution is detailed elsewhere (Mrak and Phaff 1948; Scott 1957; Ingram 1957; Onishi 1963; Spencer 1968; Pitt 1975; Tilbury 1980a,b).

Several expressions have been used to describe yeasts able to thrive in low water activity environments. These include osmophilic (Richter in 1912 cited in Onishi 1963; Christian 1963 ; Scarr and Rose 1966), osmotophilic (van der Walt 1970), osmotoduric (van der Walt 1970), osmotolerant (Anand and Brown 1968), and osmotrophic (Sand 1973). However, Brown (1976) recommended the use of the term "xerotolerant" to explain yeasts growing at low water activities (see Section 1.3.1.1). The term "xerophilic" is used to describe those yeasts that require low water activities .

1.3.1 Growth characteristics

Water stress affects several phases of growth and multiplication of yeasts. When a yeast inoculum is transferred to a culture with low a_w, there usally is a lag phase; the length of which increases with decreasing a_w (Phaff et al. 1952; Ross and Morris 1962; Nokrans 1966; Wei et al. 1982; Horner and Anagnostopoulos 1973). In most situations, the exponential growth rate (Nokrans 1966; Anand and Brown 1968; Watson 1970) and biomass yield (total mass of yeast per unit volume of growth medium) (Tanner et al. 1981; Wei et al. 1982) are lower at the lower a_w.

Effects of water stress in yeast viability can be found in articles by Edgley and Brown (1983), Onishi and Shiromaru (1984) Morris et al. (1986). In common, their results suggest that the lag phase which is usually encountered by adaptation of yeast to a water stress, is accompanied by a catastrophic decrease in viability. The extent of the drop in viability is a function of the yeast species, magnitude of the stress, and the solute used to adjust the a_w of the stress culture and the plating agar.

1.3.1.1 Nature of the solutes

The generalization made by Scott (1957) that the growth response of many microorganisms to a particular a_w is by and large independent of the solute used to adjust the a_w does not seem to be true for many yeast species. In fact, the nature of the solutes that lowers the a_w of a culture determines the minimum a_w value for a yeast. For example, when *S. rouxii* is grown in media in which sugars lower the

a_W value, it stops growing when the value reaches around 0.60. However, when salts lower the a_W value, the minimum value for growth is around 0.85. Further examples of this response of yeast are detailed in Onishi (1963). Moreover, Anand (1969) found one strain of *S. rouxii* , which did not grow in a medium when sucrose was used to lower the a_W value to 0.85, but did grow in the same medium at 0.80 a_W when a supplementary addition of glycerol was used to lower further the a_W value. In recognition of the importance of the nature of solutes, Brown (1976) subdivided xerotolerant yeast into sugar tolerant (e.g. *S. rouxii*) and salt tolerant (e.g. *Debaromyces hansenii*).

The degree of tolerance of low water activities is a characteristic of individual species or strains. An example of this difference in tolerance can be found from work by Anand and Brown (1968). Five strains of *S. cerevisiae* (nonxerotolerant) were tested for their response to low water activities adjusted with either polyethylene glycol or sucrose. Four of the five strains could not grow at a_W value of 0.895 or below when sucrose lowered the a_W. When the a_W value was increased to 0.917, four of the five strains did grow with the exception of strain Y41 which only grew at a higher a_W (0.935). When polyethylene glycol was used to lower the a_W value, none of the strains could grow below 0.93 a_W and one of the strains (Y43) could not grow below 0.95 a_W value.

The different responses of a yeast to different solutes may be attributed to the environment from which it was isolated. For instance, *S. rouxii* isolated from marzipan was unable to grow in a medium containing 3.5 M NaCl whereas, many strains isolated from shoyu mashes and miso pastes can tolerate growing at more than 3.5 M NaCl (Mori and Windisch 1982).

1.3.1.2 Xerotolerant, nonxerotolerant and xerophilic yeasts

It is difficult to make a precise definition of xerotolerance since, as we have seen, water relations of yeasts vary with the solute used to adjust a_W (Brown 1976). Previously, the term "osmophilic" introduced by Richter in 1912 (see Onishi 1963)

was used to explain the same group of yeasts that is now referred to as xerotolerant (Brown 1976). However, Brown (1976, 1978a) commented on the semantic problem of using such a term, firstly because of the connotation that the prefix "osmo-" implies and secondly because its suffix "-philic" refers to a requirement for, rather than a tolerance of, a concentrated solution. Hence, the term "xerotolerant" was proposed to cover this entire group of microorganisms (e.g. xerotolerant fungi, xerotolerant yeasts) (Brown 1976). Xerotolerant yeasts were then subdivided into sugar- and salt tolerant (see Section 1.3.1.1). The general biology of xerotolerant yeasts can be found in articles by Ingram (1957), Scott (1957), Onishi (1963) Pitt (1975), and Tilbury (1980 a,b). Predominant xerotolerant yeasts are *Debaromyces hansenii*, *Hansenula anomola*, *Pichia omeri*, *Saccharomyces bailii* var. *osmophilus* (now known as *S. rouxii*), *S. bisporus*, *Schizosaccharomyces pombe*, *Torulopsis candida* (*Candida famata*) and *T. lactis-condensi* (*C. lactiscondensi*) (Tilbury 1980 a,b). The most common species of this group is *S. rouxii* . This species is a common contaminant in sugar refineries and other industries that process concentrated solutions of sugars.

Nonxerotolerant yeasts indicate nontolerance of, or sensitive to, low a_w or high solute concentration. Good examples of these yeasts are *S. cerevisiae* and *S. fragilis* , *S. cerevisiae* being a sensitive yeast. Details about the general response to solute stress of this species can be found in various articles such as Phaff et al. (1952), Brown (1976, 1978a), Edgley and Brown (1983) and Mackenzie et al. (1988).

Xerophilic yeasts are those yeasts that requires low a_w or high solute concentration. An example from this group is *Torulopsis halonitratophila* . Onishi (1960) reported that this yeast is obligately halophilic at 30°C and facultatively so at 20°C. Accordingly, the yeast was first isolated from a culture containing 18 % (m/v) NaCl. Then it was trained by serial transfer down to 6 % NaCl. When the salt concentration reached 3 % NaCl, this species grew with difficulty after a long lag phase, and did not grow at all in lower concentration. All of these were performed at 30°C. However at 20°C, *T. halonitratophila* grew in a dilute medium without supplementary addition of salt. Nonelectrolytes can have a similar effect (Brown 1978a).

Increasing temperature may enhance tolerance of high solute concentrations (Onishi 1963; Brown 1964) and this phenomenomenon has been observed in osmophilic yeasts (Gibson 1973; Corry 1976) and species such as *S. bailii* and *S. cerevisiae* (Wilson et al. 1978). A study by Anand and Brown (1968) on the xerophilic yeast *Zygosaccharomyces nectarophilus* found that at 30°C, this yeast required a low a_w when polyethylene glycol was used to adjust the a_w. However, this yeast can grow in a dilute medium (0.997 a_w) at temperatures ranging from 16-23°C (Margaret Edgley unpublished results cited by Brown 1978a).

1.3.1.4 Preconditioning (acquired tolerance)

Nontolerant yeasts can be "trained", "preadapted", or "preconditioned" to improve tolerance against a sudden transfer to low a_w. That is to say, a nontolerant yeast unable to survive transfer to, say a specified a_w can be preconditioned by exposure to an intermediate level of a_w (e.g. Mackenzie et al. 1986). Normally, when mid-exponential phase cells of *S. cerevisiae* are transferred to salt broth , one cell in 10^4 survived plating on low water activity agar due to 'water stress plating hypersensitivity' (Mackenzie et al. 1986, 1988; Mackenzie 1988). However, this discrepancy was eliminated by preconditioning cultures i in 2% (m/v) or more NaCl. This adaptation of the *S. cerevisiae* culture was accompanied by an accumulation of glycerol. The amount of intracellular glycerol that accumulated in response to growth in 2% NaCl (about 0.99 a_w) was substantially less than that needed for complete osmotic adjustment to synthetic honey agar (0.924 a_w). This suggests that the protection conferred by glycerol was achieved through its role as compatible solute rather than by the osmoregulatory process of complete turgor adjustment (Mackenzie et al. 1986).

In addition, the xerotolerance in microorganisms can be lost by prolonged cultivation in a dilute medium; and restored again by "training" (transferring the yeast through a series of increasing solute concentration) (see Ingram 1950; Scarr 1951; English 1954; Bellinger and Larher 1986; Onishi 1957, 1963).

1.3.2 Intracellular composition

As described in Section 1.2.3, a microorganism must accumulate a compatible solute(s) to tolerate growth in low a_w environment. However, in order to adapt to a change from one water activity to another it must osmoregulate. This is accomplished by regulating the content of one or more osmoregulatory solutes (Brown et al. 1986). Although glycerol is the only compatible solute so far identified in eukaryotes to perform this role under extreme conditions (Brown 1978a), other solutes are known to have increased with low a_w.

1.3.2.1 K^+ and Na^+

Microorganisms growing in low a_w environment encounter problems caused by increase ionic strength. Adaptations of halophilic and halotolerant microbes to these conditions can be divided into insulation, protection, and modification (Reed 1986). Insulation is a type of adaptation which reduces the influence of the external environment upon the cell interior (e.g. entry of salt). Protection deals with securing intracellular function and the metabolic activity from the inhibitory effect of sodium ions (Na^+). Modification is concerned with those aspects of cellular metabolism which show optimal function in high salt conditions. These three categories of adaptation are not mutually exclusive and can be envisaged as "form of defence" against salt stress, with insulation acting as the first defensive barrier and modification providing the last reserve of the organism (Reed 1986).

High concentrations of Na^+ are generally toxic to most of the processes which occur within living cells (Brown 1976; Wyn Jones and Gorham 1983). Thus most bacteria, fungi and algae possess membranes with a lower permeability to Na^+ than K^+ and an active Na^+ extrusion mechanism (Harold 1977; Raven 1980; Jennings 1983). Yeast is no exception. Halotolerant yeast *D. hansenii* and nonxerotolerant *S. cerevisiae* regulate their intracellular salt compositions by maintaining higher K^+ : Na^+ ratios than in the medium (Nokrans and Kylin 1969). A similar mechanism seems to occur in the algal genus *Dunaliella* (Borowitzka and Brown 1974).

It is interesting to note that *D. hansenii* accumulates both K^+ and glycerol intracellularly as the concentration of the salt medium increases (Nokrans and Kylin

1969). This may plausibly show that at least two or more solutes can change with a_w and with the phase of the growth (Brown 1978a).

1.3.2.2 Glycerol

Yeasts generally accumulate polyols (polyhydric alcohols) or related compounds in response to a lowered water activity. Under extreme conditions the major polyol accumulated is glycerol (Brown 1978a). Three major functions of the accumulated intracellular polyols have been discussed by Brown (1978a), namely: (a) osmoregulation, (b) 'food' storage, and (c) protection. Function (c) is indispensable since, it is axiomatic that, in order for an organism to grow, its enzyme complement must be functional (Brown 1976, 1978a). If enzymes are to remain functional at low levels of a_w, cytoplasmic solutes should not be excessively inhibitory. Microorganisms can achieve this in two ways: (i) produce enzymes that are inherently resistant to high solute (especially salt) concentration, or (ii) form an intracellular environment that is not excessively inhibitory. As in Section 1.2.3, extremely halophilic bacteria use both methods whereas, eukaryotes are generally restricted to modification of their interiors by accumulating compatible solutes.

In addition, a high accumulation of glycerol and other polyols (such as erythritol, arabitol and mannitol) has been found in all xerotolerant yeasts isolated from environments containing high sugar concentrations when cultures were grown in 60% guccose medium (Spencer and Sallan 1956). It was demonstrated that low environmental water activity (i.e. high sugar or salt concentration) is the major environmental factor that increases polyol production in xerotolerant yeasts (Onishi 1963; Spencer 1968; Spencer and Spencer 1978). When the intracellular component of xerotolerant and nontolerant yeasts in high a_w was analysed, xerotolerant yeasts contained one or more polyols whereas no detectable polyol was found in the nontolerant yeasts (Brown and Simpson 1972; Brown 1974). However, when grown in low water activities, glycerol was accumulated in proportion to external a_w by xerotolerant *Debaromyces hansenii* (Gustafsson and Nokrans 1976; Adler and Gustafsson 1980), *S. rouxii* (Brown 1978a; Edgley and Brown 1978) and *Hansenula* sp. (Ozawa annd Twamoto 1981) and the nonxerotolerant *S. cerevisiae* (Brown 1978a; Edgley and Brown 1978). The time course of glycerol accumulation in *S. rouxii* and *S. cerevisiae* during adaptation has been studied

(Edgley and Brown 1983).

There are important physiological differences in the means by which glycerol content is controlled in the two species. *Saccharomyces cerevisiae* produces more glycerol in response to a lowering of a_w but retains a constant proportion of the solute within the cell. This energetically wasteful method has been called "the American method" (see Brown 1978a). On the other hand, *S. rouxii* is energetically conservative, i.e. it responds to diminished low a_w by varying the proportion of glycerol it retains while producing an approximately constant amount of glycerol. Therefore, glycerol content of *S. cerevisiae* and *S. rouxii* are respectively regulated metabolically and at the level of permeability or transport (Brown 1978a). In addition, it is perhaps this energetically wasteful method used by *S. cerevisiae* in accumulating glycerol that explaining its relative intolerance of diminished water activities.

Furthermore, *Debaromyces hansenii* has been shown to accumulate glycerol in log phase and arabinitol in stationary phase in response to high salinity (Adler and Gustaffson 1980; Nobre and Da Costa 1985). Arabinitol was the most dominant intracellular solute throughout the growth cycle in low salinity. Arabinitol also accumulate in *S. rouxii*.

1.3.2.3 Trehalose

Trehalose (alpha glucopyranosyl alpha-D-glucopyranoside), a non-reducing disaccharide, is widely distributed in the spores of yeast and many other fungi (Thevelein 1984 a,b). It also occurs in bacteria, algae and lower plants, insects and invertebrates (Elbein 1974; Thevelein 1984 a,b). This disaccharide functions, together with glycogen, as an energy reserve in yeast (Panek 1963; Panek and Matoon 1977; Lillie and Pringle 1980) and has also been proposed to function as a regulator of glycolytic flux (Panek 1985). Recent findings indicate that trehalose also acts both as a protectant of membrane stability during freezing and desiccation (Crowe et al. 1984; Carpenter et al. 1986; Oda et al. 1986; Crowe et al. 1987) (see also Section 1.4.2).

Trehalose was earlier reported to accumulate in exponential phase cells of *S. cerevisiae* in response to low a_w when adjustment of low a_w value was

made either by sodium chloride or polyethylene glycol (Margaret Edgley, unpublished results cited in Brown 1978a). *Saccharomyces rouxii* produced only a trace amount of the disaccharide and the content did not respond significantly to water stress (Margaret Edgley,unpublished results cited in Brown 1978a). In addition, the trehalose content of *S. cerevisiae* was pronounced during the adaptation period of a mid-exponential phase culture to salt broth (10 %, m/v, NaCl). Indeed, trehalose was detectable by paper chromatography of cell extracts from 20 h onwards during the response of *S. cervisiae* to salt stress. None was identified in paper chromatograms of the xerotolerant *S. rouxii*..

A recent report by Mackenzie et al. (1988) suggested that trehalose might be a more effective protective agent, per mole, than glycerol against "water stress plating hypersensitivity" (see Brown et al. 1986; Mackenzie et al. 1986, 1988; for detail of this phenomenon).

1.4 HEAT SHOCK INDUCTION OF THERMOTOLERANCE IN YEASTS

When an organism is exposed to a sudden increase in temperature (heat shock) or even other environmental stresses, it responds by synthesizing a number of highly conserved proteins called heat shock proteins (hsps) (see Ashburner and Bonner 1979; Schlesinger et al. 1982; Sanchez and Lindquist 1990) that function by inducing thermotolerance (Lindquist 1986). Whereas a number of studies report on the protective role of hsps in thermotolerance (see above), others report on the protective role of trehalose in conferring thermotolerance (Grba et al. 1975; Hottiger at al. 1987; Attfield 1987). Recent review articles on several aspects of the heat shock response are found in Neidhardt et al. (1984), Lindquist (1986), Bienz and Pelham (1987), Bond and Schlesinger (1987) and Rothman (1989).

1.4.1 Heat shock proteins

The response of cells to heat shock was first described by Ritossa (1962) when he found a new set of puffs on the salivary gland chromosomes of a fruitfly, *Drosophila buskii*, induced by heat, dinitrophenol, or sodium salicyclate. Subsequent work showed that the puffs were: (i) induced by several other stress

treatments (Ritossa 1962; Berendez 1968; Ashburner 1970; Leenders and Berendez 1972), (ii) produced within a few minutes (Berendez 1968; Ashburner 1970), (iii) associated with newly synthesized RNA (Ritossa 1962; Leenders and Berendez 1972), (iv) found in other *Drosophila* species and in many different tissues (Ritossa 1962; Berendez 1965), and (v) associated with the disappearance of the previously active puffs (Berendez 1968; Leenders and Berendez 1972). This was later shown to be a result of active gene transcription resulting in the formation of a small set of proteins. For more than a decade, this selective induction of proteins by a heat shock was thought to be unique to *Drosophila* species. Since 1978, hsps were reported as a result of heat shock in avian and mammalian tissue culture cells (Kelly and Schlesinger 1982), and others found similar activity in *E. coli* (Lemeaux et al. 1978; Yamamori et al. 1978), Tetrahymena (Guttman and Gorovsky 1979) and yeasts (McAlister et al. 1979; Miller et al. 1979).

Heat shock proteins can be defined by two criteria: (1) their formation is intensively stimulated by an environmental stress; in particular, that resulting from an increase in temperature of a few degrees centigrade, and (2) their genes contain 14 base pairs in the 5' noncoding region (Schlesinger 1986). At present, hsps are found in virtually all organisms - from *E. coli* to man (Schlesinger et al. 1982).

It is a natural and widely held assumption that the purpose of the heat shock response is to protect organisms from the destructive effects of heat and other forms of stress. Heat shock proteins are induced in different organisms at different temperatures, but in each case, the temperatures correspond to the upper region of organism's natural growth range. The induction of the production of hsps coincides with the acquisition of tolerance to more extreme temperatures. Studies by Sanchez and Lindquist (1990) resulted in isolation of a heat shock protein gene, HSP 104, from *Saccharomyces cerevisiae* and a deletion mutation was introduced to the yeast cells. They found that mutant cells grew at the same rate as the wild type cells and died at the same rate when exposed directly to high temperatures. However, when both strains were given a mild pre-heat treatment, the mutant cells did not acquire tolerance to heat, as did wild type cells. Transformation with the wild type gene counteracted the defect of mutant cells. Their results showed that a particular heat shock protein plays a critical role in cell tolerance at extreme temperatures. Other heat shock proteins of the HSP 70 family which are found in bacteria and cytoplasm, mitochondria, and the lumen of the endoplasmic reticulum of eukaryotic cells are

described in a recent review by Rothman (1989). On the other hand, thermotolerance has been reported to occur without any detectable level of hsps. For example, maize (*Zea mays*) seedlings when preconditioned prior to heatshock with either a progressive water stress (water stress was achieved by placing seedling roots in a shallow solution of increasing water potential) of -0.25 megapascal/ hour from 0 to -1.25 megapascal over a 6-hour time period, or exposure to various concentrations of salts. These seedlings showed an induced thermotolerance to high temperature (either 40 or 45°C) although no induced hsps were detected (Bonham-Smith et al. 1987). Furthermore, maize which did accumulate hsps due to preconditioning at 2°C/h from 26 to 36°C tolerate a severe water stress up to -2.0 megapascals although these plants were no better protected against water stress than those preconditioned with a progressive increase in solute concentrations which did not produce hsps (Bonham-Smith et al. 1987).

Some conflicting results in the role of hsps in the induction of thermotolerance was found in yeast (*S. cerevisiae*). For example, when *S. cerevisiae* grows at 37°C, it synthesizes hsps and becomes more thermotolerant. However, aminoacid analogues that are potent inducers of hsps failed to induce thermotolerance, suggesting that the stress proteins do not play a casual role in aquired thermotolerance at 37°C. Thus, Hall (1983) suggested that the acquired thermotolerance might have been caused by the reaction of a pre-existing cellular component which was activated at 37°C or by the inactivation at 37°C of a component that prevented thermotolerance.

In general, resistance to elevated temperature is probably mediated not only by the synthesis of hsps, but also by other mechanisms as well (Linquist 1986). Polyols (polyhydroxy carbohydrates and derivatives) (Henle et al. 1982) and trehalose (Attfield 1987; Hottiger et al. 1987) are examples of those agents that can protect cells against heat as well as desiccation (see Section 1.4.2).

Attfield (1987) discussed the possibility that heat shock proteins might share the attributes of acting in the normal cell growth and multiplication , as well as protector against stress. Heat shock system affecting dynamics of the relationship between the *Drosophila melanogaster* genome and one of its retrotransposons, copia, has been studied by Strand (1988). He shows that copia is regulated in various ways by the host genome: (i) copia is subject to control by the host-encoded heat shock system,

(ii) formation of copia RNA is regulated during development (iii) copia is expressed tissue specifically during development, and (iv) intracellular localization of copia follows a specific pattern of different tissues. In addition, an insertion of a copia element into the *adh* gene reduces the *adh* transcript abundance and disrupted normal developmental promoter usage. The mechanism by which this transposon insertion altered *adh* expression involves not only displacement of cis-acting controlling elements, but also interference by copia element expression. Since transposable elements are also found in yeast, also as a major source of mutation, it is possible to suggest that 45°C preincubation induces production of heat shock proteins together with trehalose to indirectly protect any source of mutation, either by a transposable element, heat or salt stress. The effects of heatshock proteins and trehalose must be indirectly through the mitochondrial DNA. It is also possible that glycerol is involved; thus, suggesting that compatible solutes and heat shock proteins share the role as protector against stress heat stress. The protective nature of glycerol in heat stress must be of compatible solute nature.

In addition, trehalose is also synthesized by *E. coli* in a minimal medium in response to water stress (Strom et al. 1986) with the trehalose content varing in proportion to the salt concentration. In a complex medium, members of the betaines (including proline betaine, choline and glycine betaine aldehyde) are predominant in *E. coli* as osmoregulatory solutes (Strom et al. 1986). Trehalose is not synthesized (Perroud and Le Rudulier 1985). Thus, trehalose is the major osmoregulatory solute in absence of osmoregulatory betaines (Larsen et al. 1987).

Escherichia coli mutants that are defective in various pathways of the trehalose synthesis have been studied (Strom et al. 1986). These mutants are osmotically sensitive to minimal medium because they are defective on some genes that code for some of the precursors of trehalose synthesis. One mutant has *gal* U mutation. This mutation blocks the function of the gene that encodes the formation of glucose-1-phosphate uridylyltransferase. As a result, the mutant fails to synthesize UDP glucose which is a precursor of trehalose synthesis, nor can it utilize galactose as a substrate (*Gal-*). Another *E. coli* mutant called the Tn 10 mutant is also unable to accumulate trehalose in response to water stress (H. Giover and Strom unpublished results, cited in Strom et al. 1986). This mutant may be defective in a gene that codes for one of the enzymes required for trehalose synthesis which is presumably trehalose-6-phosphate synthase. However, when the betaines are added

to the minimal medium, these mutants are no longer osmotic sensitive (H. Giover and Strom unpublished results, cited in Strom et al. 1986). It is clear from these studies that trehalose is a reserve solute that cells use as a protector against stress.

1.4.2 Trehalose and acquired thermotolerance

Trehalose has been reported to accumulate in yeast during exposure to agents that induce heat shock response (Grba et al. 1975; Hottiger et al. 1987; Attfield 1987). Hottiger et al. (1987) found that the trehalose content of exponentially growing cells of *S. cerevisiae* increased progressively in response to a rise in temperature from 27 to 40°C, and decreased again when the temperature was returned from 40 to 27°C. These changes were accompanied with increases and decreases in the thermotolerance and desiccation tolerance of the cells. Similar results were found by Attfield (1987). In addition, Attfield (1987) found that various agents such as ethanol, copper sulphate, and hydrogen peroxide induced trehalose accumulation. However, the heat shock induction of the disaccharide requires de novo RNA synthesis. Incubating growing cells in the presence of inhibitors of RNA synthesis such as acridine orange and ethidium bromide resulted in a massive inhibition of trehalose accumulation (Attfield 1987).

The pattern of trehalose accumulation in response to a temperature shift shows a similarity to the synthesis of heat shock proteins in yeast. For instance, trehalose is present in a large amount in cells entering stationary phase or those starved of nutrients such as nitrogen, phosphorus or sulphur (see Lillie and Pringle 1980). The disaccharide is also stored in ascospores and is required when these reproductive structures germinate (Thevelein 1984; Panek and Bernadez 1983). On the other hand, hsps are also present in cultures progressing toward stationary phase and during sporulation (Kurtz et al. 1986; Petko and Lindquist 1986). Hence, trehalose and heat shock proteins are also likely to play a role in normal cell growth and multiplication as well as protectors against stress (Attfield 1987; Hottiger et al. 1987).

1.5 AIMS OF STUDY

There was a suspicion from earlier studies (Professor A.D. Brown, personal communication) that there was an alteration in colonial morphology when a mid-exponential culture of *S. cerevisiae* was transferred to a new liquid nutrient medium containing 10 % m/v NaCl. These morphological effects were ill-defined and their existence was apparently transient in transfer experiments of this kind. In addition, Edgley and Brown (1983) had compared *S. rouxii*, a xerotolerant yeast, and *S. cerevisiae* in a number of aspects of their responses to a salt stress. The results showed in essence that *S. rouxii* adapted readily to the salt stress whereas *S. cerevisiae* did so only with great difficulty. For example, the changes in viability of *S. cerevisiae* were characterized by two stages of adaptation. Stage 1 was marked by a catastrophic drop of the apparent viability and different counts on plating media of different water activity. Stage 2 was characterized by similar counts on the various plating media and progressive increase in viability.

The results of this project demonstrated that the previous ill-defined morphological effects were *petite* colonies (see Section 1). Furthermore, up to 35% *petite* mutants were found following transfer of *S. cerevisiae* strain Y41 in mid-exponential phase to salt broth compared with about 1% that arose spontaneously in the normal culture before transfer. On the other hand, a culture which was harvested from stationary phase did not give rise to a significant increase in the proportion of the mutants.

A previous project (Petelo, BSc Hons Thesis; 1985) confirmed the results of Edgley and Brown (1983) for *S. cerevisiae* . In addition, the highest incidence of *petite* mutants was found in Stage 2 phase of adaptation rather than immediately after the stress in Stage 1. Accordingly, two questions were then proposed to explain the mechanism of this phenomenon : Either the increase in the proportion of *petite* mutants in the Stage 2 phase of adapatation was (1) a result of a greater resistance of the pre-existing *petite* strains to salt stress, or, (2) a mutagenic action by the solute (salt) stress.

The present thesis describes research which was primarily to distinguish between those two possible mechanisms.

CHAPTER TWO : MATERIALS AND METHODS

MATERIALS AND METHODS

2.1. MATERIALS :

2.1.1. Growth media and chemical reagents

The following chemical reagents and components of growth media were used :

Name	Grade A.R. - Analytical Reagent	Manufacturer
Acetic Acid	A.R.	BDH
Agar		OXOID
Anthrone		SIGMA
Bacteriological Peptone		OXOID
Benzoic Acid		SIGMA
Calcium Chloride ($CaCl_2.2H_2O$)	A.R.	MERCK
Difco Yeast Extract		OXOID
Ethanol	A.R.	AJAX
Glucose	A.R.	AJAX
Glycerol	A.R.	MAY&BAKER
Magnesium Sulfate($MgSO_4.7H_2O$)	A.R.	BDH
Malt-extract Agar(MA)		OXOID
Potassium Dihydrogen Orthophosphate (KH_2PO_4)	A.R.	AJAX
Sodium Chloride (NaCl)	A.R.	AJAX
Sulphuric Acid	A.R.	AJAX
Trehalose		SIGMA
2,3,5-Triphenyl Tetrazolium Chloride		SIGMA

2.1.2 Organisms

One wild-type and two mutant strains of *Saccharomyces cerevisiae* were used during this project. *Saccharomyces cerevisiae* , strain Y41, American Type Culture Collection (ATCC) number 38531, described by Anand & Brown (1968) as non-xerotolerant was the wildtype strain. The mutant yeasts; a glycogen-deficient mutant strain 212-244-1A, defective at the *glc* 1 locus (Pringle, 1972) and, a temperature-sensitive cell division cycle mutant, strain 182-6-3, cdc-24-1 (Hartwell *et al.*, 1973) were purchased from the Yeast Genetic Stock Center (University of California, Berkley).

2.1.3 Stock cultures

The organisms were maintained on malt-extract agar (MA) slopes (Edgley and Brown, 1983). Wildtype and glycogen-deficient mutant strains took about 2 days to form colonies on MA at 30°C, whereas, the temperature sensitive mutant took 4-5 days. The temperature sensitive mutant will not grow at 37° C but does grow at 30°C or below. The stock cultures, all in screw-capped bottles, were kept at 4° C and subcultured every 2-3 months.

2.1.4 Liquid growth medium

Experimental cultures were grown in basal yeast medium (BYM, a conventional broth) (Edgley and Brown 1983). The composition of the BYM is :

Peptone	5.0 g
Yeast extract	2.5 g
KH_2PO4	1.0 g
$MgSO_4.7H2O$	0.25 g
$CaCl_2.2H2O$	0.1 g
Glucose	2.0 g

Distilled water to 1 litre.

pH	6.1
a_w	0.997

The water activity (a_w) of this medium was adjusted to lower values by addition of appropriate amounts of NaCl; such as for the 'adapted or pre-conditioning medium' (2% m/v, NaCl; a_w 0.985) and for 'Salt Broth' (10% m/v, NaCl; a_w 0.936).

Media were autoclaved at a pressure of 83 kpa for 12mins.

2.2 GENERAL METHODS :

2.2.1 Culture conditions

Organisms were grown in a rotary shaker at 30°C, unless stated otherwise, and 200 r.p.m. The first stage was inoculated using 1-2 loops of yeast from a yeast slope to a 100 ml Erlenmeyer flask containing 50 ml BYM. It was incubated until the cells reached stationary phase of growth. The second stage was initiated with a 1% inoculum, by volume, from the first stage culture in BYM (or BYM containing 2% NaCl where appropriate) into a total volume of 100 ml in Erlenmeyer flasks, there being 2-3 times the volume of the medium. These cultures were grown to mid-exponential phase before a 10% inoculum, by volume, was used to inoculate the experimental stage (salt broth, 100 ml; BYM, 100 ml) unless stated otherwise with the aim to initiate this culture between 10^4 to 10^5 yeast per ml as determined by a microscopic count (see Section 3.2.2).

Strains Y41 and 212-244-1A took about 2.5-3 h to reach mid-exponential phase at the second stage whereas, the temperature sensitive strain cdc 24 took about 4-6 h. The *petite* isolates always took a little longer to grow than their normal counterparts. Stationary phase cultures were prepared by allowing second stage cultures to inoculate overnight or for 24 - 36 h as specified.

2.2.2 Pre-conditioning experiments

The stages of culture used for pre-conditioning experiments were the same as tin 3.2.1 above unless stated otherwise.

In 2% (m/v) NaCl pre-conditioning, all second stage cultures were grown in BYM

containing 2% (m/v) NaCl to mid-exponential phase before being transferred into the experimental medium.

In 45°C preconditioning, the whole second stage culture at mid-exponential phase (100 ml) was exposed to a water bath equilibrated at 45°C and 200 r.p.m. for 36 mins prior to transfer into either salt broth or BYM (at 52°C) unless otherwise stated. These heatshocked cultures took 7 mins in the 45°C water bath to reach 45°C.

2.2.3 Determination of the cell number

Cell counts (total microscopic count) were made using a haemocytometer (an improved Neubauer counting chamber; Weber, England). Samples for colony and microscopical count were first homogenised in a glass/teflon homogeniser (diameter, 16mm) to disaggregate clumps. A sample of approximately 3 ml was homogenised by 75 double strokes of the teflon plunger (about 2 min). A cell or an aggregation of cells that would, if viable, produce a single colony was called a colony forming unit (CFU). Budded cells (CFU that had a bud or buds) were expressed as a percentage of total CFU's. Mid-exponential phase was considered to have been reached when 80% of the cells were budding.

2.2.4 Plating media, viable count and serial dilutions.

Malt Extract Agar was used routinely for plate counts by a drop plate method. Serial dilutions were made in quarter strength Ringer's solution supplemented with NaCl (2% or 10 %, m/v) as required to match the modified growth media.

All viable counts were made at least in duplicate; and when counts fell below 10 colonies (per 0.1 ml), several plates were used. All plates were incubated at 30°C, unless otherwise stated.

2.2.5 Testing and isolation of *petite* mutants.

Petite mutants, used for screening experiments, were detected as small colonies by observing plate cultures in a laminar flow cabinet with the aid of a stereo-microscope and a grid. The grid was made simply by photocopying a metric

ruler and cutting this to the size of the agar plates. Thus, the diameter of the *petite* colony isolated for screening was always ≤1mm.

In addition, the following confirmative procedures were used as appropriate.

(a) Where possible *petite* colonies were tested by overlaying plate cultures with TTC (2,3,5-triphenyl tetrazolium chloride) according to Lindegren et al. (1958). This involved dissolving 1mg/ml of TTC in molten 1.5% Bacto agar and adjusting the pH to 7.0 with M/15 phosphate mixture.This agar was cooled to 50°C , poured over the diagnostic plate cultures and incubated at 30°C. TTC is a dye that reacts with the metabolically active yeast giving the normal or *grande* colonies a red (formazan) colour within an hour while the small or *petite* (respiratory deficient) colonies remained white. *Petite* isolates for death rate experiments were initially streaked to confirm their stability and also tested by overrlaying with TTC. *Petite* colonies of the temperature sensitive mutant cdc 24 were easy to score as they are always white whereas, the normal *grande* colonies were red-pink (see Pearson et al. 1986). Pigmentation of the *grande* colonies is due to *ade* 1 and *ade* 2 mutations (Pearson et al. 1986).

(b) The other method used to identify *petites* was streaking. Small colonies which were assumed as *petite* colonies were identified microscopically and isolated under a laminar flow cabinet (see above). A MA petri-dish was divided in half; the *petites* were streaked on one side and the normal or *grande* colony on the other half. The plate was incubated at 30°C for 2 days in case of strains Y41 and 212-244-1A and 4-5 days in case of strain 182-6-3, before being examined for *petites*. Small colonies with stable size (< 1mm) and always small in comparison to the normal colonies were considered *petite* mutants.

Since these confirmative procedures for identifying *petite* mutant phenotype were reliable, no further work was done to confirm *petite* phenotype by plating on a mediun containing a nonfermrntable substrate, such as glycerol, in which the true *petites* should not grow.

2.3 ANALYTICAL METHODS

2.3.1 Preparation of Extracts for Analysis

Samples (100ml) of relevant cultures were immediately centrifuged at 10,000 g for 2mins. The supernatant was decanted and the pellets were suspended in distilled water to 5ml. Part of this suspension (2 x 1ml) was used for the determination of dry mass and the other portion (2ml out of 3ml) was centrifuged. This supernatant was decanted. The pellets were suspended in water to 2 ml and centrifuged again. The supernatant solution was collected and immediately frozen. The pellets were then extracted with absolute ethanol (2 ml) according to Edgley and Brown (1983) and allowed to stand overnight at 0°C. This ethanolic slurry was centrifuged and washed twice in the centrifuge with water (1 ml). The extract and washings were combined and frozen in the liquid nitrogen before being freeze-dried. This freeze-dried powder was suspended in 1ml distilled water.

2.3.2 Trehalose Estimation

Trehalose was estimated with anthrone according to Stewart (1975) using 0.2 ml cell extract to 1 ml anthrone.

2.3.3 Glucose Estimation

Glucose was estimated enzymically with a commercial glucose oxidase (EC 1.1.3.4, GOD-Perid kit Boehringer, Germany) according to Edgley & Brown (1983).

2.2.4 Determination of the Dry Mass

Dry mass was determined on the yeast suspensions by drying at 80-100° C for 24h and cooling in a dessicator before weighing the dry mass.

CHAPTER THREE : RESULTS

3.1 THE EFFECT OF SALT STRESS ON THE VIABILITY OF *SACCHAROMYCES CEREVISIAE* AND THE PRODUCTION OF*PETITE* MUTANTS.

Mid-exponential stage two cultures of yeasts were used for inoculation of the experimental flasks containing salt broth (10% m/v, NaCl). The effect of salt stress on the viability and the proportion of *petite* mutants of *Saccharomyces cerevisiae* was determined by plating samples at various incubation times on MA. After 48 h of incubating plate cultures, which was folded on plastic bags and incubated at 30°C, the total number of viable colonies was counted and the number of *petite* colonies was initially detected in a laminar flow cabinet with the aid of a stereo-microscope and a grid (see MATERIALS & METHODS). Other confirmative procedures for identifying *petites* were used as indicated. The total viable counts the number of viable *petites* were expresssed in logarithmic scale. The proportion of *petite* colonies are mainly expressed as percentage of the total viable count throughout this thesis. All experiments were repeated at least in duplicates. Three strains of *S. cerevisiae* were used in this investigation (see Materials and Methods). In particular, a temperature sensitive mutant 182-6-3 (cdc 24) was initially employed to test the reproducibility of the salt-induced *petite* mutagenesis (Petelo 1985) among other strains of *S. cerevisiae*

Figure 3.1a shows changes occurred in the viability of *Saccharomyces cerevisiae* strain 182-6-3 (cdc 24) in the experimental medium containing salt broth, inoculated from stage two culture harvested from mid-exponential phase in BYM. There was a drop of some 100-500 fold in the total viable count in the first 48 h, after which it recovered to the original level. Both *petite* and normal cells were killed in the salt broth. The total microscopic count did not drop, however. Reductions in viable counts were also observed. This reduction is clearly shown by strains cdc 24 and 212-244-1A (Figures 3.1a & 3.1c) whereas, in strain Y41, the drop in viability reached its lowest count at 24 h. After that, the viability increased. The drop in viability of strain Y41 has been previously observed by Edgley and Brown (1983), Petelo (1985) and Mackenzie (1988). However, the production of *petite* mutants accompanying this phenomenon was not observed until Petelo (1985).

The decrease in total viable count was accompanied by a change in the proportion of *petites* . Within each strain there is an increase in the proportion of *petite* mutants after 24h. The increase in the proportion of *petite* mutants was more pronounced in

strains Y41 and 212-244-1A (*glc* 1), 17.6 and 26.5 fold respectively (Figures 3.2b & 3.2c) than for cdc 24 (Figure 3.2a) where the increase was 3.75 fold.

Table 3.1 provides the statistical analysis of the change in numbers of mutants obtained with three strains of *Saccharomyces cerevisiae*, Y41, 182-6-3 (cdc 24) and 212-244-1A (*glc 1*) when exposed to salt stress. Strain Y41 had a 17 and 27 fold increase at 24 h and 72 h respectively in the proportion of *petite* mutants. Strain 182-6-3 (cdc 24) had about 2-fold increase at 24 h after which it gradually declined to about the same level as existed before the stress. The proportion of mutants was greatest in strain Y41 after 72 h but occurred considerably earlier with the other two strains. The highest proportion and proportional increase of *petites* was obtained with strain 211-244-1A (*glc 1*) where, after 24 h *petites* accounted for almost half the population.

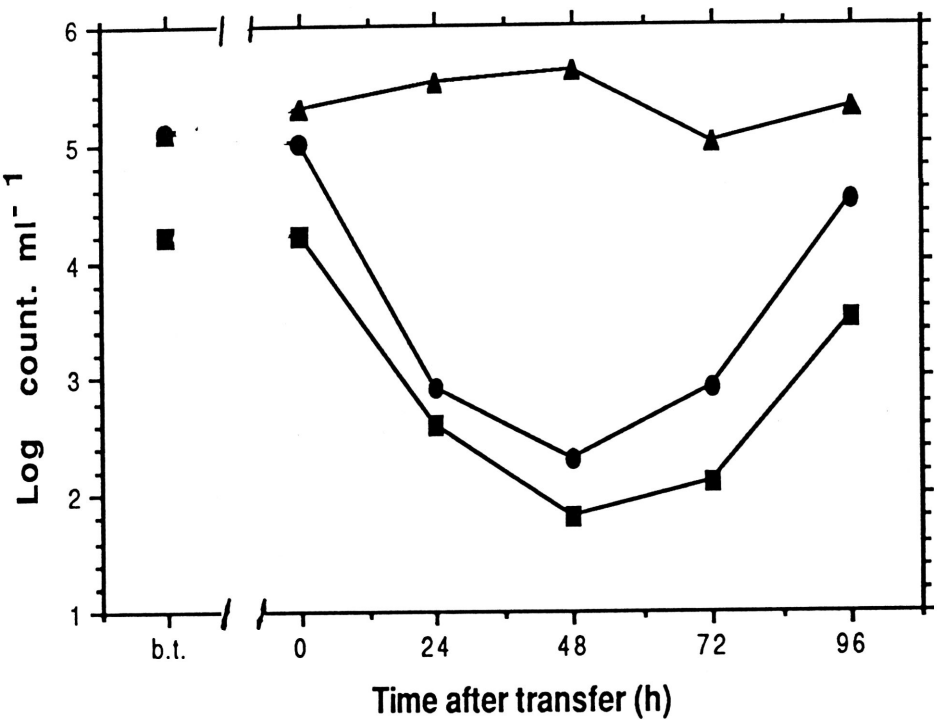


Figure 3.1a Changes in the total viable and *petite* population of Saccharomyces cerevisiae strain 182-6-3 (cdc 24). Mid-exponential phase stage two cultures (10% v/v) in BYM were used to inoculate the experimental flasks containing salt broth.

Total colony count (●); *petite* colony count (■); total microscopic count (▲).

The "before transfer" (bt) sample was taken immediately before inoculation of the salt broth.

Petites were identified by colony sizes under stereo-microscope. These petites were then streaked on MA before being overlayed with TTC.

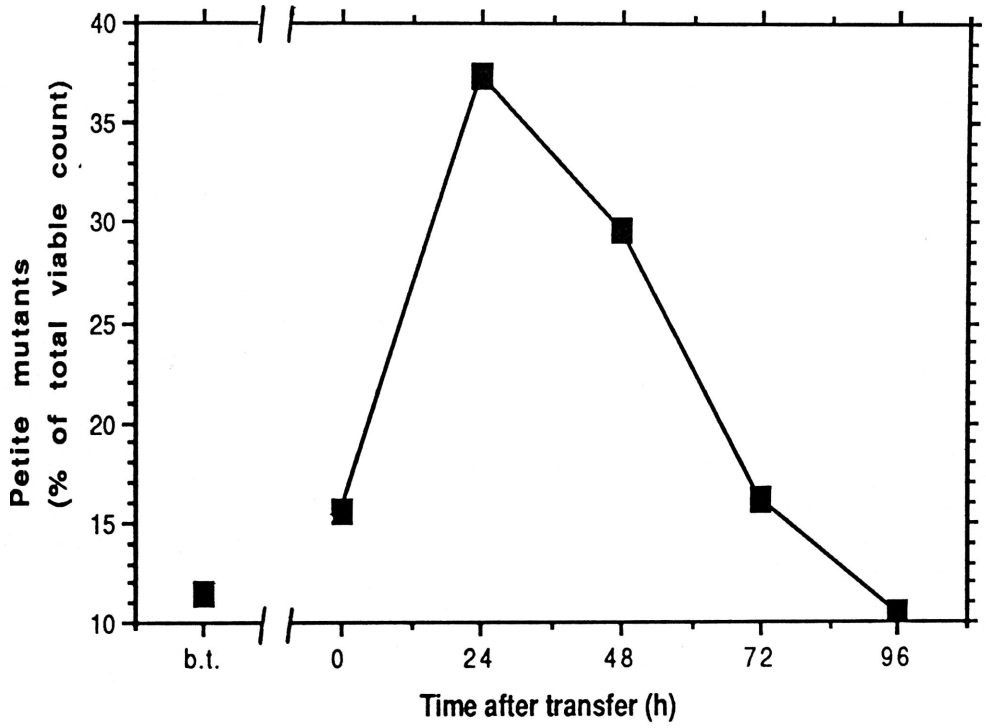


Figure 3.2a The proportion of *petite* mutants (%) in the same culture as in Fig 3.1a.

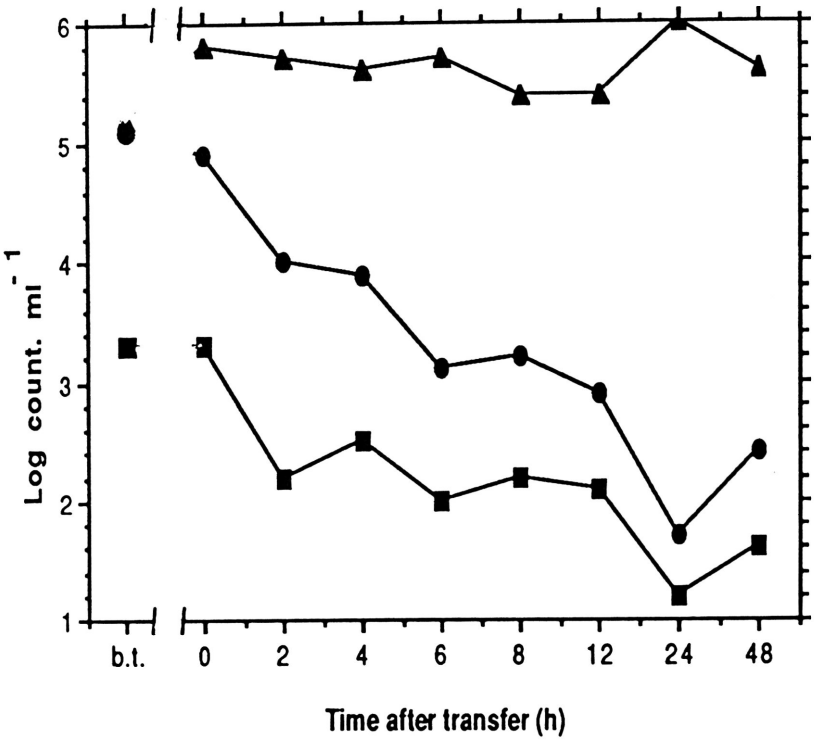


Figure 3.1b Changes in the total viable and *petite* population of Saccharomyces cerevisiae strain Y41. Mid-exponential phase stage two cultures (10% v/v) in BYM were used to inoculate the experimental flasks containing salt broth. Total colony count (●); *petite* colony count (■); total microscopic count (▲).
The "before transfer" (bt) sample was taken immediately before inoculation of the salt broth.
Petite mutants were identified by colony sizes under stereo-microscope and further tested by streaking on MA.

Note the time scale.

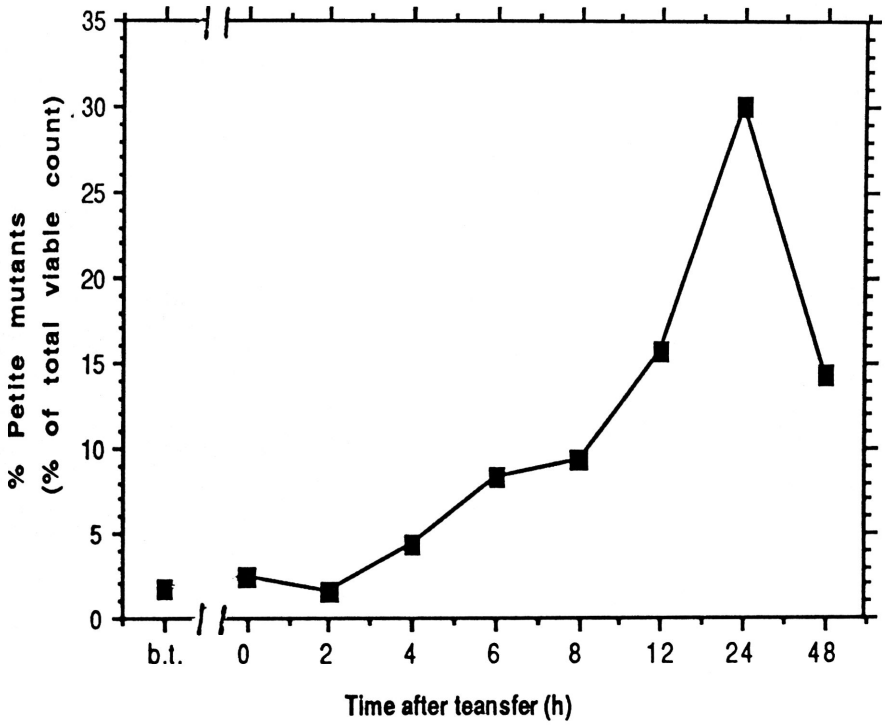


Figure 3.2b **The proportion of *petite* mutants (%) in the same culture as in Figure 3.1b.**

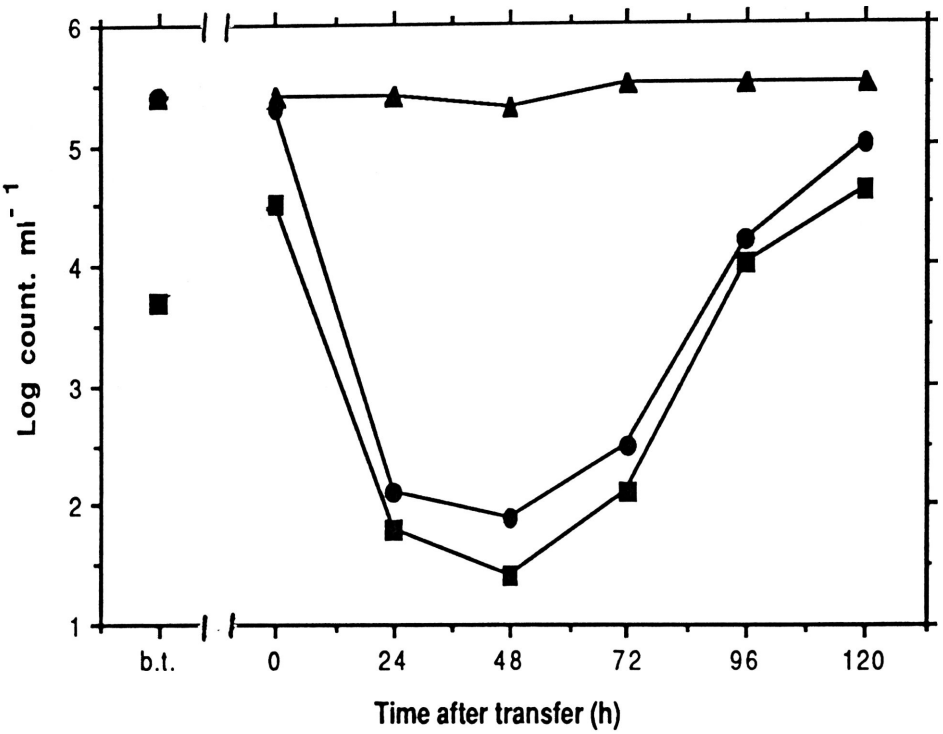


Figure 3.1c Changes in the total viable and *petite* population of Saccharomyces cerevisiae strain 211-244-1A (*glc* 1). Mid-exponential phase stage two cultures (10% v/v) in BYM were used to inoculate the experimental flasks containing salt broth.

Total colony count (●); *petite* colony count (■); total microscopic count (▲). The "before transfer" (bt) sample was taken immediately before inoculation of the salt broth.

Petite mutants were identified by colony sizes under stereo-microscope.

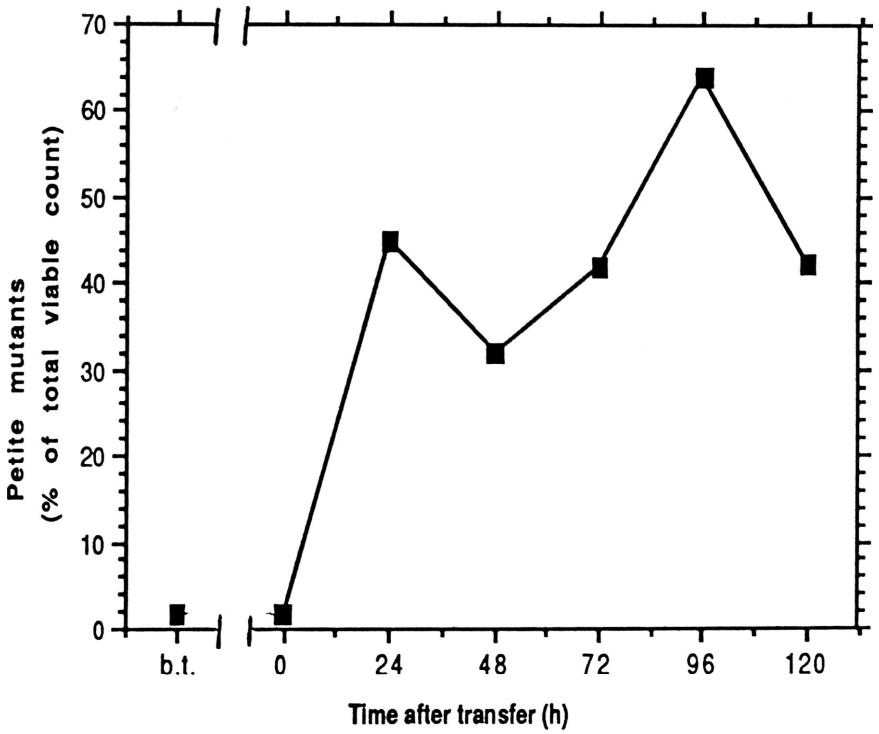


Figure 3.2c **The proportion of *petite* mutants (%) in the same culture as in Figure 3.1c.**

54

TABLE 3.1. **The effect of salt broth on viability of *Saccharomyces cerevisiae* and the proportion of *petite* mutants.**

Strains							
		Time after transfer (hours)					
	b.t*	0	12	24	36	48	72
Y41							
Proportion of Petite Mutants (%)	1.3	2.7	-	22.0	-	29.3	34.8
SDM	±0.6	±1.1		± 8.4		±9.5	±8.5
	[7]	[6]		[2]		[5]	[12]
LVC	5.2[b]	5.1		2.3		2.5	3.3
SDM	±0.2	±0.1		±0.1		±0.2	±0.3
182-6-3 (cdc 24)							
Proportion of Petite Mutants (%)	17.2	20.4	22.3	30.5	28.0	19.5	16.2
SDM	±6.7	±4.0	±3.0	±12.2	±8.2	±11.4	±2.4
	[10]	[10]	[8]	[8]	[5]	[9]	[2]
LVC	5.2[b]	4.5	3.7	2.8	2.3	2.3	2.9
SDM	±0.1	±0.3	±0.1	±0.2	±0.1	±0.1	±0.0
211-244-1A (*glc 1*)							
Proportion of Petite Mutants (%)	1.5	3.2	-	46.1	-	40.6	37.7
SDM	±0.9	±1.5		±6.8		±15.1	±12.3
	[4]	[4]		[4]		[4]	[8]
LVC	5.5[b]	5.4		2.4		2.0	2.7
SDM	±0.1	±0.1		±0.2		±0.1	±0.2

TABLE 3.1

SDM is Standard deviation of the mean; LVC is Log viable count (Mean of logs for number of yeast suspensions).

All strains were grown at 30°C (see MATERIALS and METHODS). Experimental conditions were as for Figures 3. 1. The numbers denote the mean and standard deviation of the proportion of petite mutants (% of the total viable count) and the corresponding log viable count. ml-1 (mean of logs for number of yeast suspensions), designated by LVC, the number of yeast suspensions analysed is shown in square brackets, this arising from at least repeated experiments, with replicate samples being analysed.

Identification of petite mutants was performed in this order; (i) *petites* were isolated under stereo- microscope, (ii) *petites* were streaked on MA, and (iii) *petite* colonies which tested positive on size criteria were overlayed with TTC (see Materials and Methods).

* Immediately before transfer to salt broth

3.2 RESPONSE OF *PETITE* ISOLATES TO SALT STRESS.

Due to the enhanced proportion of *petite* mutants arising after transfer of second stage mid-exponential culture to salt broth (Section 3.1; Petelo 1985), it was considered that the mechanism of this phenomenon might be due to either mutagenesis or greater resistance of the *petite* population observed before the culture was transferred to the salt broth experimental inoculum. Thus, the response of *petite* colonies isolates to the salt stress was investigated. The *petite* colonies isolates and the parental (normal or *grande* colonies) (see Section 2.2.4) were grown in BYM, and 10% v/v mid-exponential phase second stage culture was used for inoculation of the salt broth. The response of *petite* colonies isolates compared to normal *grande* colonies to salt stress was determined by viable count on MA taken during the first 24 h; this period the yeast cells are under enormous stress as shown by drop in the viable count (Section 3.1).

Experiments for which the results are provided could not indicate the mechanism by which the proportion of *petites* in the yeast population changed after the salt stress. That is to say, distinguishing between mutagenesis and greater resistance of pre-existing *petites* was not possible. In order to resolve this question, *petite* strains were isolated and their death rates in salt broth were compared with those of the parent strain.

Notwithstanding the relatively small increase in the proportion of *petites* observed with cdc 24 strain, comparative measurements of death rate were initially made in this strain using TTC (Section 2.2.4). With this strain, normal *grande* colonies had a slight red (formazan) colour, whereas the *petites* were white. Accordingly, 10 cdc 24 *petite* colonies were isolated before the salt stress and 9 *petite* colonies were isolated 48 and 72 h after the stress were exposed to the same experimental treatment as in Section 4. 1 and their response compared with that of the parent cdc 24 strain Figure 3. 3 shows representative survival curves for the cdc 24 parent and one *petite* strain.

Table 3. 2a shows the mean and standard deviation of the log viable count / ml of the parent strain cdc 24, as well as the *petite* colonies isolated before and at 48 h and 72 h after transfer to salt broth. The results indicate that *petite* strains died at least as fast as the parent strains in the salt broth. Thus there was no enhanced

survival of *petite* strains in salt broth suggesting that the increase in the proportion of *petite* mutants in 'Stage 2' was due apparently to the mutagenic action of the salt stress.

Repeating the experiments using *petite* colonies isolated from strain Y41 generally supported this conclusion (see Table 3.2b), although death rates were determined only at 0 and after 8 h. Intermediate colonies of Y41 which were qualitatively stable, as assessed by stereo-microscope, after salt stress were screened together with the *grande* (wild type) and *petites* before/ after stress isolates. Tests of significance ("students" t-test) of the difference in mean of the death rates (Log count at 0 h - Log count at 8 h) of the wild type versus the other isolates showed that they were not significantly different. Intermediate colonies were not only observed in Y41 after salt stress, but also on other strains, although no further studies were undertaken. In all other experiments, the intermediate colonies were classified as *grandes* as they were red with TTC.

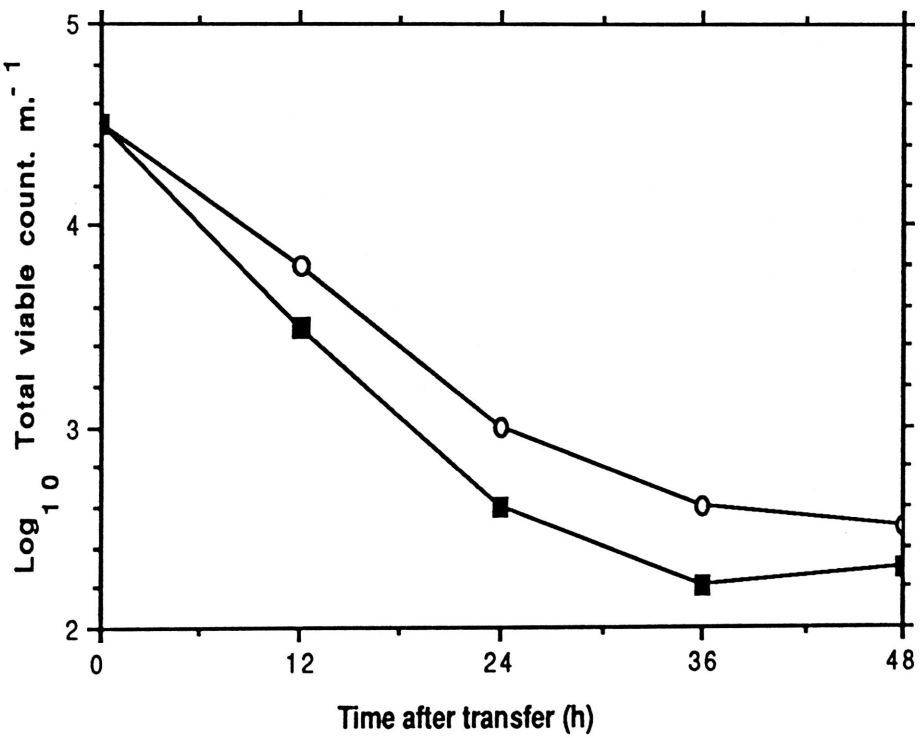


Figure 3.3. **Survival curves of cdc 24 parent (O) and one petite isolate (■) in salt broth. The mid-exponential phase stage two cultures (10% v/v) of the parent and petite colonies isolates were used to inoculate experimental flasks containing salt broth.**

TABLE 3.2a. Viability count of *Saccharomyces cerevisiae* strain 182-6-3 (cdc 24) and its *petite* colonies isolates on exposure to salt stress.

Strain 182-6-3 (cdc 24)					
				Difference*	
	0	12	24	12	24
	at sampling time (h)			at sampling time (h)	
Parent strain					
Log count.ml-1	4.8	3.5	2.8	1.3	2.0
SDM	±0.5	±0.5	±0.6	±0.7	±0.9
No. of samples	[5]	[5]	[5]	[5]	[5]
Petite colonies isolated before stress					
Log count.ml-1	5.1	3.0	2.3	2.1	2.8
SDM	±0.1	±0.5	±0.4	±0.3	±0.4
No. of samples	[9]	[9]	[9]	[9]	[9]
Petite colonies isolated after stress					
Log count.ml^{-1}	4.8	3.3	2.4	1.5	2.4
SDM	±0.3	±0.4	±0.8	±0.5	±0.8
No. of samples	[10]	[10]	[10]	[10]	[10]

All cultures were harvested from the second stage mid-exponential phase culture prior to screening in the experimental flasks containing salt broth. Experimental conditions were as in Figure 3.1 or Table 3.1.

* (Log count at 0 h - log count at 12 h or 24 h).

Petite colonies attained after stress were isolated at random from plates at 48 h and 72 h. Identification of petite mutants was performed in this order; (i) petites were isolated under stereo- microscope, (ii) petites were streaked on MA, and (iii) petite colonies which tested positive on size criteria were overlayed with TTC (see MATERIALS and METHODS).

TABLE 3.2b. Viability count of *Saccharomyces cerevisiae* Y41 wild type, intermediate normal colonies and *petite* colonies isolates on exposure to salt stress.

Strain Y41	Time After Transfer (Hours)		Differences in Log
	0	8	8 h
Wild type			
Log count.ml^{-1}	4.8	2.6	2.2
SDM	±0.3	±0.8	±0.9
No. of samples	(11)	(11)	(11)
Petite colonies isolated before stress			
Log count.ml^{-1}	4.9	2.3	2.0
SDM	±0.4	±0.7	±0.7
No. of samples	(16)	(16)	(16)
Petite colonies isolated after stress*			
Log count.ml^{-1}	4.7	2.4	2.2
SDM	±0.5	±1.1	±1.2
No. of samples	(10)	(10)	(10)
Intermediate colonies isolated after stress			
Log count.ml^{-1}	4.9	2.4	2.5
SDM	±0.4	±0.6	±0.6
No. of samples	(10)	(10)	(10)

All cultures were harvested from the second stage mid-exponential phase culture prior to screening in the experimental flasks containing salt broth. Experimental conditions were as in Figure 3.1 or Table 3.1.

* (Log count at 0 h - log count at 8 h).

Petite and intermediate colonies colonies attained after stress were isolated at random from plates at 48 h and 72 h. Identification of petite mutants was performed in this order; (i) *petites* were isolated under stereo- microscope, (ii) *petites* were streaked on MA, and (iii) *petite* colonies which tested positive on size criteria were overlayed with TTC (see MATERIALS and METHODS).

3.3 FACTORS AFFECTING THE PROPORTION OF *PETITE* MUTANTS ARISING AFTER TRANSFER TO SALT BROTH.

3.3.1 Age of Culture.

The *petite* mutation rate is considered to be sensitive to the age of a culture. As a culture ages the tendency to generate *petites* decreases (Wallis and Whittaker 1974). Exponential cultures are generally more sensitive to physical stresses than are stationary phase cultures. Hence, experiments were undertaken to determine the changes of viability and proportion of *petite* mutants when a second stage culture of *S. cerevisiae* strain 182-6-3 (cdc 24) in BYM was incubated to stationary phase before being used for inoculation of the experimental salt broth.

Table 3.3 shows viable count and the proportion of *petite* mutants in some stationary phase cultures of *Saccharomyces cerevisiae* strain cdc 24 at selected time intervals after transfer to salt broth. Judging from the percentage of budded cells (Table 3.3), about two thirds of the yeast population were in the G1 stage (see Hartwell 1974) of the cell division cycle at zero time (t_0). Unlike exponential phase cultures, which were killed, stationary phase cultures did grow in salt broth after a lag phase (Fig. 3.4).

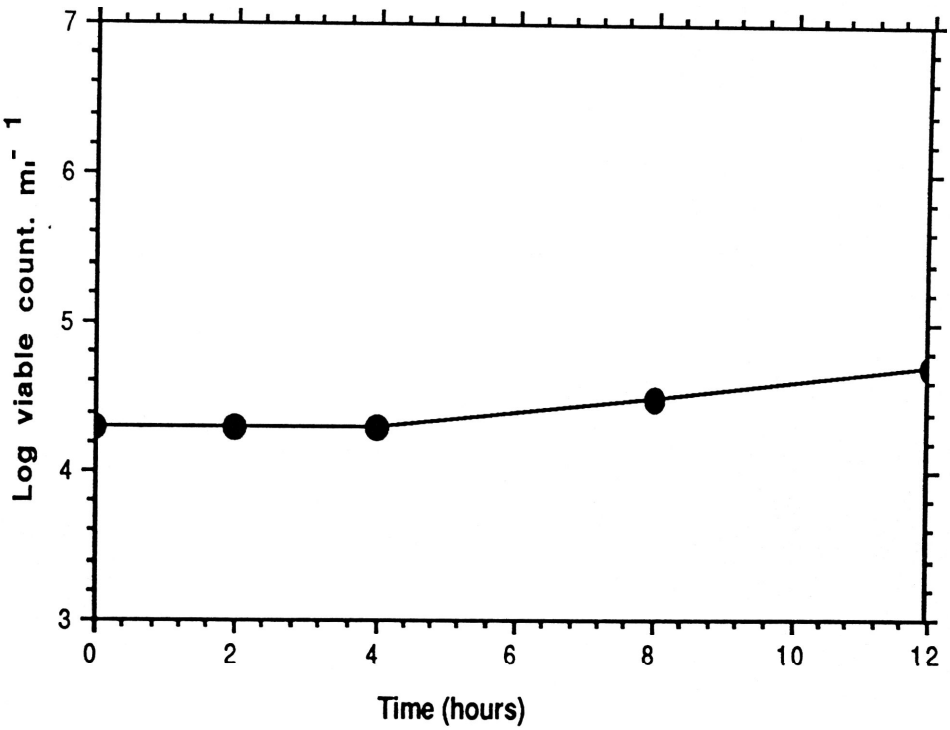


Figure 3.4. Changes in the viable count when a second stage culture of Saccharomyces cerevisiae strain 211-244-1A (cdc 24) in BYM were grown to stationary phase before 10% v/v was used to inoculate experimental flasks containing salt broth.

TABLE 3.3 **Viability of *petite* mutants arising from response of stationary phase cultures of *Saccharomyces cerevisiae* strain 182-6-3 (cdc 24).**

Time After Transfer (h)	Log (Total Viable count/ml)	Log (*Petite* count/ml)	*Petite* Mutants (% of total viable count)	Budded Yeast Cells (% of CFU)
0	5.4	4.6	15.9	32.0
12	5.7	4.9	15.9	12.7
24	6.3	5.4	12.6	9.1

The data represent the average of two experiments. Second stage cultures of *Saccharomyces cerevisiae* 182-6-3 (cdc 24) were incubated with shaking at 30°C for 30 h to reach stationary phase prior to transfer into the experimental flasks containing salt broth.

Petites were tested with TTC.

3.3.2 Pre-conditioning in 2% NaCl (glycerol accumulation).

Mild stress generated by growth of *Saccharomyces cerevisiae* in 2% (m/v) NaCl stimulates sufficient glycerol accumulation to confer complete protection against the lethal effects of plating into 8% NaCl or SHA (see Mackenzie et al. 1986). Accordingly, the effect of this treatment on the viability of the yeast on transfer to salt broth and on the frequency of the stress-induced *petite* mutation was investigated.

All cultures were grown in the second stage to mid-exponential phase in 2% (m/v) NaCl before being used to inoculate experimental flasks containing salt broth. Table 3.4 shows the result of such preconditioning for strains Y41, 182-6-3 (cdc 24), and 211-244-1A (*glc 1*); viable counts being determined at selected time intervals. Comparative data from Table 3.1 for cultures not pre-conditioned with 2% NaCl is included in TABLE 4.4. The screening of Y41 preconditioned cultures was confined to 24 h and 72 h in the salt broth because, this was the period where the mutation rate was higher in non-preconditioned cultures (see TABLE 3.1). Thus, CFU at time before / immediately after transfer is included for this strain.

Looking at the number of *petites* seen at the end of the pre-conditioning period (before transfer i.e. TABLE 3.4), it can be seen that strain 182-6-3 (cdc 24) had a higher proportion of mutants than 211-244-1A (*glc 1*). This may suggest that the frequency of *petites* is a characteristic of the yeast strain itself (see for example Gilberg et al. 1967). All viable counts at 'before transfer' and 0 h were adjusted using microscopic count to start the culture between 10^4 to 10^5 yeast per ml.

When the number of *petite* mutants arising after transfer from the second stage preconditoned cultures to salt broth were assessed, all 3 strains of *S. cerevisiae* generated less *petite* mutants as compared to the number of *petites* arising from the transfer of nonpreconditioned cultures into salt broth (TABLE 3.4). During the salt stress period, the mutation rate of the temperature-sensitive strain cdc 24 was almost identical to that at the 'b.t.' 2% NaCl preconditioned period. In comparison, cdc 24 stage two mid-exponential phase non-preconditioned cultures show a significant difference in the proportion of mutants when compared with the proportions arising after transfer into salt broth. This difference is significant at the 0.1% level.

In addition, the viability at 24 h after transfer of the preconditioned cultures into salt

broth increased by 31.6% and 15.8% respectively for strains 182-6-3 (cdc 24) and 211-244-1A (*glc* 1). The viability of Y41 also increased by 25.1% at 24 h as a consequence of preconditioning. Moreover, the incidence of *petite* mutants in preconditioned cultures of strain 211-244-1A (*glc* 1) decreased at 48 h and 72 h in the salt broth as compared to cultures that were not preconditioned. This indicates that *petite* mutagenesis was suppressed in a later stage (see DISCUSSION). The differences in preconditioned and non-preconditioned cultures were significant at the 0.1% level. This may suggest that *petite* cells do not survive prolong exposure to a salt stress which may be directly or indirectly due to *glc* 1 mutation.

TABLE 3.4 **Effects of 2% NaCl preconditioning on proportion of *petite* mutants in salt broth.**

Strain	Time After Transfer (h)					
	b.t.*	**0**	**12**	**24**	**48**	**72**
Y41						
Proportion of Petite Mutants (%)		-		13.1	-	24.9
SDM				±3.6		±7.0
LVC	5.6**	5.6**		3.8		5.5
SDM	±0.0	±0.0		±0.2		±0.1
No.of suspensions	(2)	(2)		(16)		(16)
	[1.3][a]	[2.7][a]		[22.0][a]		[34.8][a]
	±0.6	±1.1		±8.4		±8.5
	[5.2][b]	[5.1][b]		[2.4][b]		[3.3][b]
	±0.2	±0.1		±0.1		±0.3
182-6-3 (cdc 24)						
Proportion of Petite Mutants (%)	27.7	28.6	27.2	31.3	-	-
SDM	±3.1	±9.	±5.4	±4.6		
LVC	[5.0]	[4.7]	[4.5]	[4.3]		
SDM	±0.1	±0.3	±0.2	±0.1		
No. of suspensions	(6)	(12)	(12)	(6)		
	[17.2][a]	[20.4][a]	[22.3][a]	[30.5][a]		
	±6.7	±4.0	±3.0	±12.2		
	[5.2][b]	[4.5][b]	[3.7][b]	[2.8][b]		
	±0.1	±0.3	±0.1	±0.2		
211-244-1A (*glc 1*)						
Proportion of Petite Mutants (%)	1.8	1.7	-	40.2	28.5	18.9
SDM	±1.0	±0.6		±6.6	±3.8	±3.02
LVC	[5.6]	[5.6]		[3.6]	[4.5]	[5.7]
SDM	±0.2	±0.04		±0.2	±0.1	±0.2
No. of suspensions	(4)	(4)		(8)	(6)	(6)
	[1.5][a]	[3.2][a]		[46.1][a]	[40.6][a]	[37.7][a]
	±0.9	±1.5		±6.8	±15.1	±12.3
	[5.5][b]	[5.4][b]		[2.4][b]	[2.0][b]	[2.7][b]
	±0.1	±0.1		±0.2	±0.1	±0.2

TABLE 3.4

Stationary phase stage one cultures of yeasts (1% v/v) were used for inoculation of the second stage culture containing 2% (m/v) NaCl and incubated to mid-exponential phase. A proportion (10% v/v) of these stage two (preconditioning) cultures was used for inoculation of the experimental flasks containing salt broth. In all cases, the size of the experimental culture was such that the viable count at t_o was between 10^4 and 10^5. Results signify the mean and standard deviation (SDM) for the number of yeast suspensions shown in parentheses. LVC designates Log viable count. ml^{-1} (mean of logs for number of yeast suspensions). Super scripts values (a & b) are corresponding values of the 'normal' (non-preconditioned second stage culture) transfer to salt broth from Table 3.1 included for comparison. They signify respectively the percentage *petite* colonies and log viable count of a 'control' suspension inoculated into salt broth without preconditioning in stage 2.

** CFU ± S.D. *Immediately before transfer to salt broth.

Identification of *petites* were the same as described in Table 3.1.

3.3.3 Temperature

The effects of temperature on salt stress-induced *petite* mutagenesis were also investigated. Experiments were originally designed to test the effect of salt stress for experimental stage at temperature lower (20°C) or higher (40°C) than the "normal" condition (30°C). It is known that some solutions of low a_w, such as media containing sucrose or sodium chloride, increase the heat resistance of osmophilic yeasts (Gibson 1973; Corry 1976) and of *Saccharomyces bailii* and *S. cerevisiae* (Wilson et al. 1978). Preliminary experiments using Y41 showed that, as in the normal condition (30°C), there was a drop in viability when mid-exponential stage two cultures were transferred to salt broth experimental media at either 20°C or 40°C. There was, however, no increase in the proportion of *petite* mutants, these being identified with TTC (Results not shown). In order to confirm this observation, strain 182-6-3 (cdc 24) was used to repeat experiments at 20°C, and TTC was used to confirm *petiite* status.

Figure 3.5 shows response of strain 182-6-3 (cdc 24) to salt stress at 20°C. Stage two cultures in BYM were grown with rotary agitation at 20°C to mid-exponential phase prior to transfer to salt broth experimental media already equilibrated at 20°C. Samples were taken at appropriate intervals to determine viable counts; four plates were used at each sampling time. Two plates were incubated at 20°C and two at 30°C (see legend in Fig. 3.5). These were carried out to test whether there were different rate of cell death and *petite* mutagenesis with respect to plate incubation temperatures. Accordingly, there was, as previously, a catastrophic decrease of viability (during the first 24 h of salt stress) after transfer of the mid-exponential cultures to salt broth. The drop in viability appeared slightly greater when assessed by plates incubated at 30°C rather than at 20°C (difference significant at the 5% level). On the other hand , there was no significant effect of plate incubation temperature on the proportion of *petite* mutants.

The decreases in viability illustrated in Figure 3.5, were associated with a 2 - 3 times increase in the proportion of *petite* mutants at both plate incubation temperatures; i.e. to say almost half the yeast cell population at 24 h was *petite* . Thereafter, there were minimal differences in viability and proportion of *petites* between plates incubated at the two temperatures, the proportion of *petite* declined eventually to reach the 'normal' unstressed level of about 15-18% as detected at 30°C.

The wild type strain Y41 and strain 211-244-1A (*glc* 1) were used to test the effect of salt stress at 52°C, the same temperature used by Schenberg-Frascino and Moustacchi (1972) (cdc 24 strain could not be used because of its temperature sensitivity). In these experiments, 28°C was used for first and second stage cultures incubation (Schenberg-Frascino and Moustacchi 1972).

Figure 3.6 shows changes in viability and the proportion of *petite* colonies of *S. cerevisiae* strain Y41, grown in BYM at 28°C to mid-exponential phase during the second stage, and subsequently inoculated into experimental flasks containing BYM already equilibrated to 52°C. Figure 3.7 shows an experiment in which a similar second stage culture was used to inoculate the experimental flasks containing salt broth equilibrated at 52°C.

Transfer to BYM at the higher temperature reduced the viability within an hour. *Petite* colonies reached their highest proportion (about 15 %) after 20 min. This is, in general, in qualitative agreement with the results of Schenberg-Frascino and Moustacchi (1972). Addition of NaCl (10% m/v) to the broth greatly diminished the death rate and lowered the proportion of mutants in the yeast suspension (Figure 3.7), extending the apparent period of mutagenesis.

Strain 211-244-1A (*glc* 1) responded similarly to Y41 in BYM at 52°C but, in this case the proportion of *petites* reached 100 % in 15 min after which it declined sharply (Fig. 3.8). In addition, the death rate was higher in strain 211-244-1A (*glc* 1) (Fig. 3.8) than in Y41 (Fig. 3.6).

Some experiments undertaken using preconditioning were extended by testing the effects of preconditioning either in 2% NaCl, in others or at 45°C was investigated. In 45°C preconditioning, the mid-exponential stage two cultures were incubated for 36 mins at 45°C before being used as inoculum for the experimental stages. Preconditioning in 2% NaCl was used for other experiments.

Figures 3.9 and 3.10 show the viability and proportion of *petite* mutants generated in experimental stage cultures grown in BYM and incubated at 52°C. Preconditioning (stage two) was undertaken by growing the strains Y41 and 211-244-1A (*glc* 1) to mid-exponential phase. Samples to estimate the viability and number of *petite* colonies were taken during the preconditioning and experimental stages. Figure 3.9 shows that the proportion of *petite* colonies of Y41 increased from about 0.01% to

2% during 36 min preconditioning at 45°C and subsequently remained relatively constant for 90 mins at 52°C. This proportion of *petites* was about 13% of the maximum attained under similar condition by yeast that had not been preconditioned at 45°C (cf. Fig. 3.6). Viability dropped by only 4-fold over 30 min at 52°C compared with 1000-fold in cultures that had not been conditioned at 45°C.

Strain 211-244-1A (*glc* 1) (Figure 3.10), on the other hand, gave a pronounced increase in the proportion of *petite* colonies during the 36 min adaptation period at 45°C. Specifically, the proportion increased from about 7% to 50% during the preconditioning period. At zero time after transfer to 52°C, the proportion of *petites* increased to about 90%. The proportion of *petites* dropped sharply, however, on further incubation at 52°C. When the culture was transferred to 52°C, the death rate of 211-244-1A (*glc* 1) was 0.2 log/30 min (Y41 in the same condition had a death rate of 0.3 log/30 min) compared to 4.1 log/30 min obtained with suspensions of *glc* 1 that were not preconditioned at 45°C (cf. Fig. 3.8). Figure 3.10 also suggests that *petite* mutants once formed have a much higher death rate at 52°C than the parent strain.

In general, the results have indicated that both strains were partially protected against death at 52°C by preincubation at 45°C. However, protection against *petite* mutagenesis was observed only in Y41.

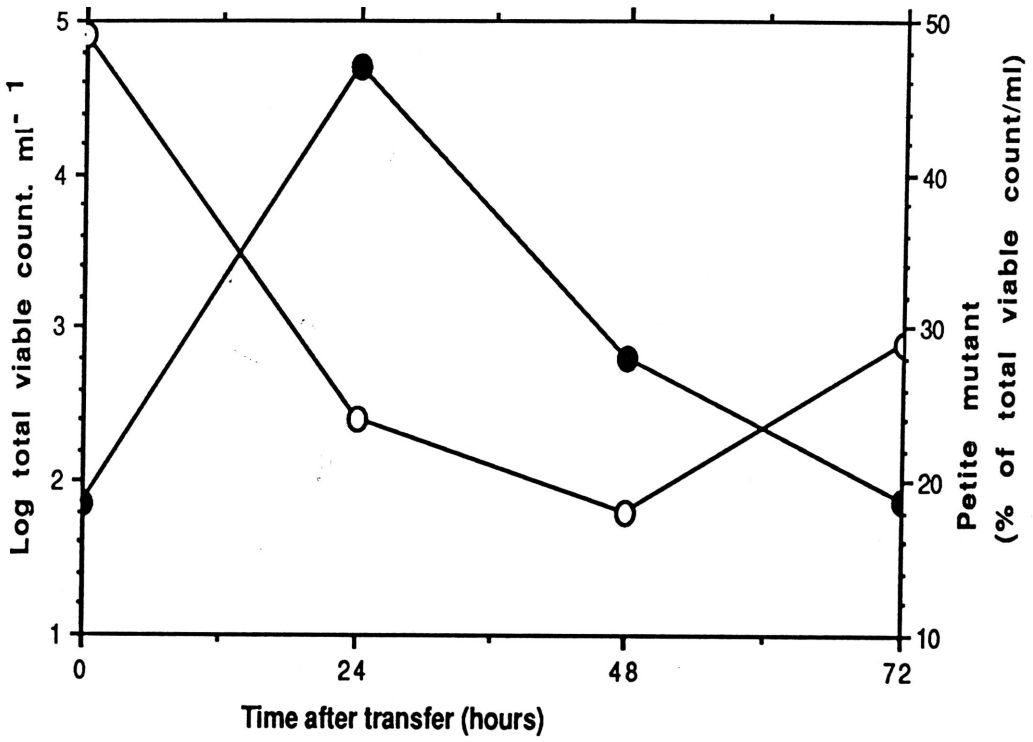
Furthermore, the cultures of strains Y41 and 211-244-1A (*glc* 1) that had been exposed in the preconditioning stage to treatment at 45°C for 36 min were used to inoculate experimental flasks containing salt broth at 30°C (as in Section 3.1). Figure 3.11 and Figure 3. 12 respectively show results obtained for viability and proportion of *petites* Y41 and 211-244-1A (*glc* 1). As previously, there was an increase in the proportion of *petite* colonies during the preincubation at 45°C.

Preincubation of the cultures of Y41 and 211-244-1A (*glc* 1) briefly at 45°C resulted in partial protection of viability when subsequently inoculated into salt broth but *petite* mutagenesis was not affected. The wild type Y41 in particular had about 30-fold decrease in the apparent viability (Figure 3.11) after 24 h in salt broth compared to 1000-fold decrease of normal culture transfer (TABLE 3.1). Similar results occurred with strain 211-244-1A (*glc* 1).

In addition, the effect of preconditioning *Saccharomyces cerevisiae* in 2% NaCl (stage two mid-exponential phase cultures) on the viability and proportion of *petites* generated by exposure to 52°C was investigated. Figure 3.13 and Figure 3.14 respectively show the effect of 2% (m/v) NaCl preconditioning in *S. cerevisiae* strains Y41 and *glc 1* . There were 100-fold and 1000-fold decreases in viability respectively for Y41 and *glc 1* after 30 min exposure to 52°C. This represents a death rate of about 10% of that obtained without the pretreatment (cf. Figures 3.6 & 3.8). Furthermore, the rate of *petite* mutagenesis was suppressed in strain Y41 but not in strain *glc* 1 . Thus, we can conclude, that partial suppression of the *petite* mutation and partial protection of viability at 52°C was observed following preconditioning in 2% (m/v) NaCl for *S. cerevisiae* Y41. Minimal protection on the viability of strain 211-244-1A (*glc* 1) obtained by this preconditioning but *petite* mutants was not affected.

FIGURE 3.5

(a) Plates incubated at 30°C.



(b) Plates incubated at 20°C.

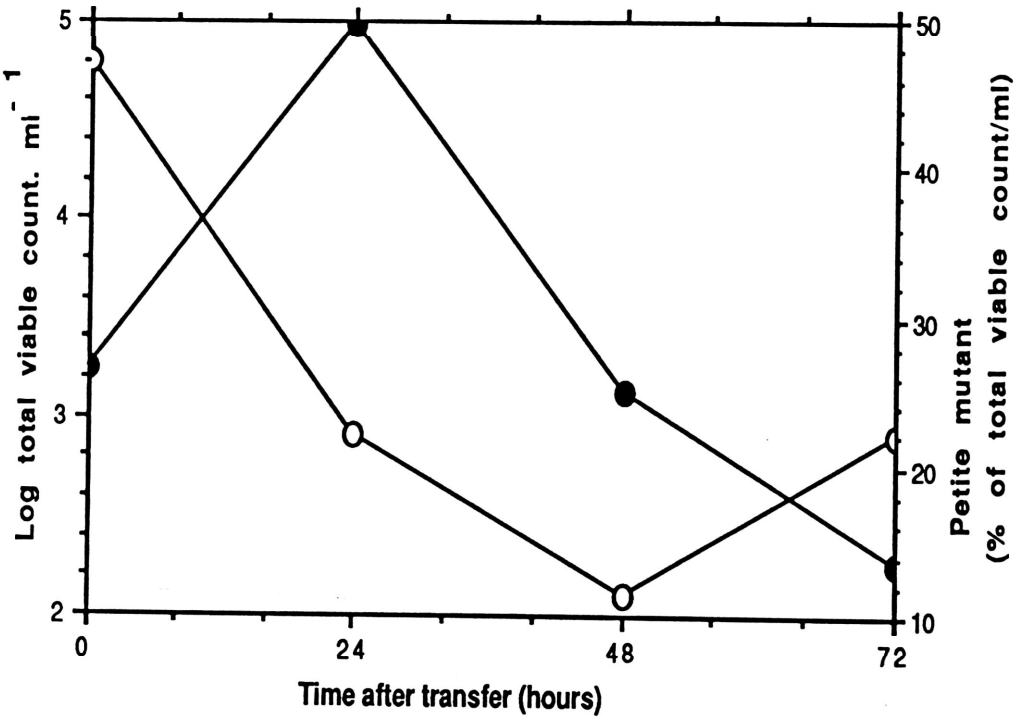


Figure 3.5

Effect of salt stress at 20°C on petite mutagenesis. Changes in the total viable and petite population of *Saccharomyces cerevisiae* strain 182-6-3 (cdc 24) after 10% v/v mid-exponential phase stage two culture in BYM, incubated at 20°C, was used to inoculate the experimental flasks containing salt broth. The experimental medium was already equilibrated at 20°C. The changes in viability (O) and the proportion of petite mutants (●) were determined on MA. Four plates were used at each sampling time; two were incubated at 30°C (a) and two at 20°C (b).

Petites were tested with TTC.

Note different time scale on the left hand axes (a) & (b).

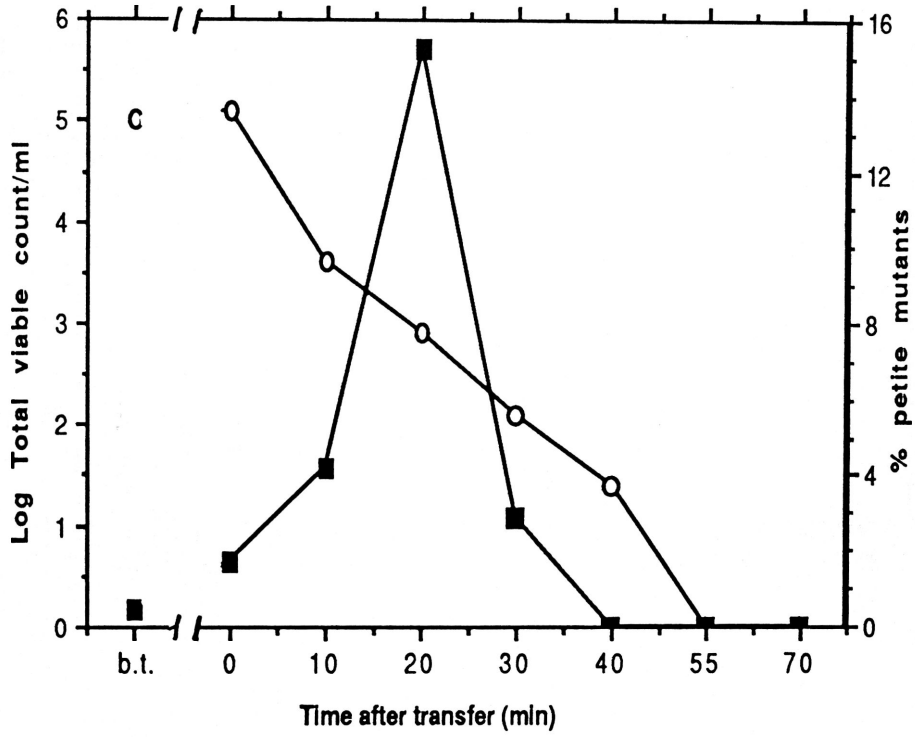


Figure 3.6 Changes in viability (○) and the proportion of *petite* mutants (■) of *S. cerevisiae* strain Y41 at 52°C. The second stage mid-exponential phase cells (10% v/v) in BYM at 28°C were used to inoculate the experimental flasks containing similar medium already equilibrated to 52°C. The "before transfer" (bt) sample was taken immediately before inoculation of the BYM. Petites were identified by colony sizes.

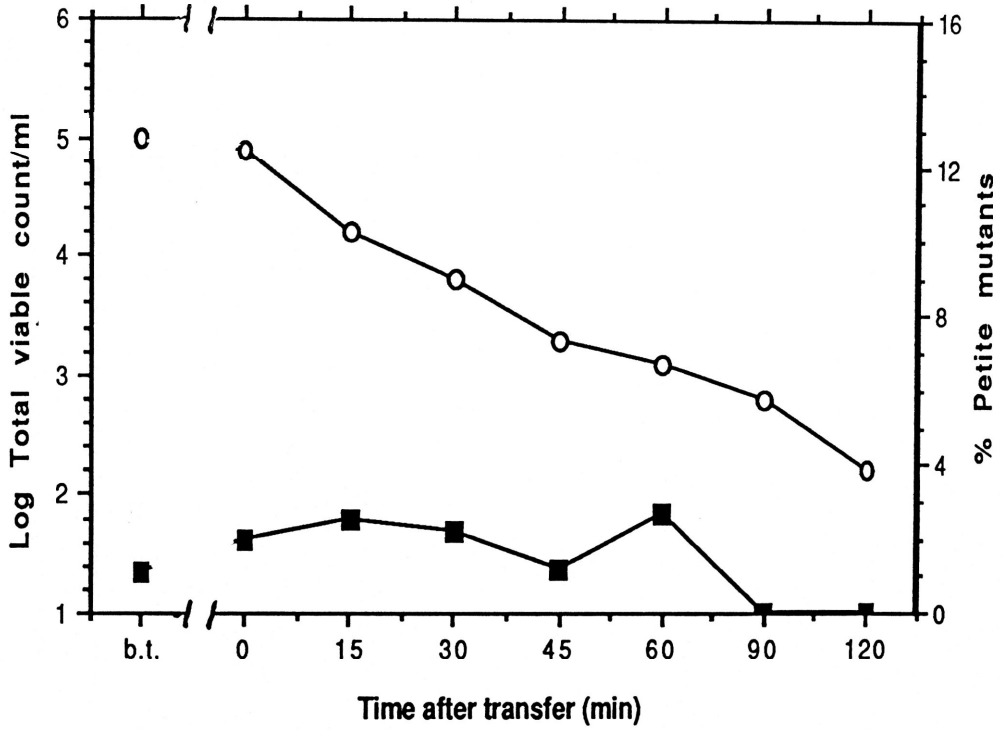


Figure 3.7 Changes in viability (o) and the proportion of *petite* mutants (■) of *S. cerevisiae* strain Y41 at 52°C. The second stage mid-exponential phase cells (10% v/v) in BYM at 28°C were used to inoculate the experimental flasks containing salt broth already equilibrated to 52°C. The "before transfer" (bt) sample was taken immediately before inoculation of the BYM. Petites were identified by colony sizes.

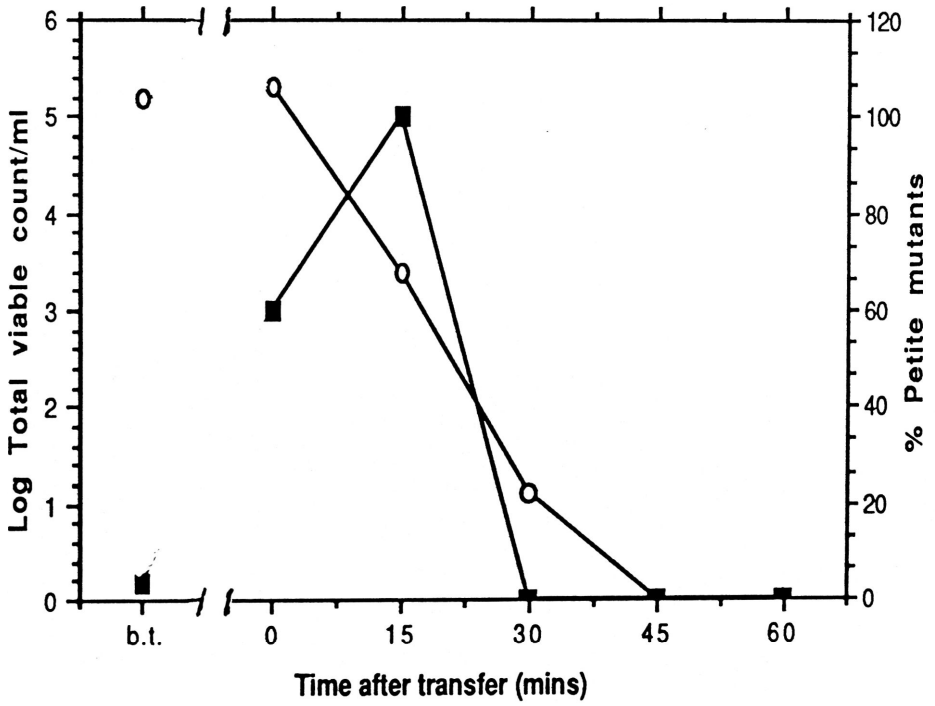


Figure 3.8 **Changes in viability (o) and the proportion of *petite* mutants (■) of *S. cerevisiae* strain 211-244-1A (*glc* 1) at 52°C (same condition as in Figure 4.6). The second stage mid-exponential phase cells (10% v/v) in BYM at 28°C were used to inoculate the experimental flasks containing similar medium already equilibrated to 52°C. The "before transfer" (bt) sample was taken immediately before inoculation of the BYM.**
Petites were identified by colony sizes.

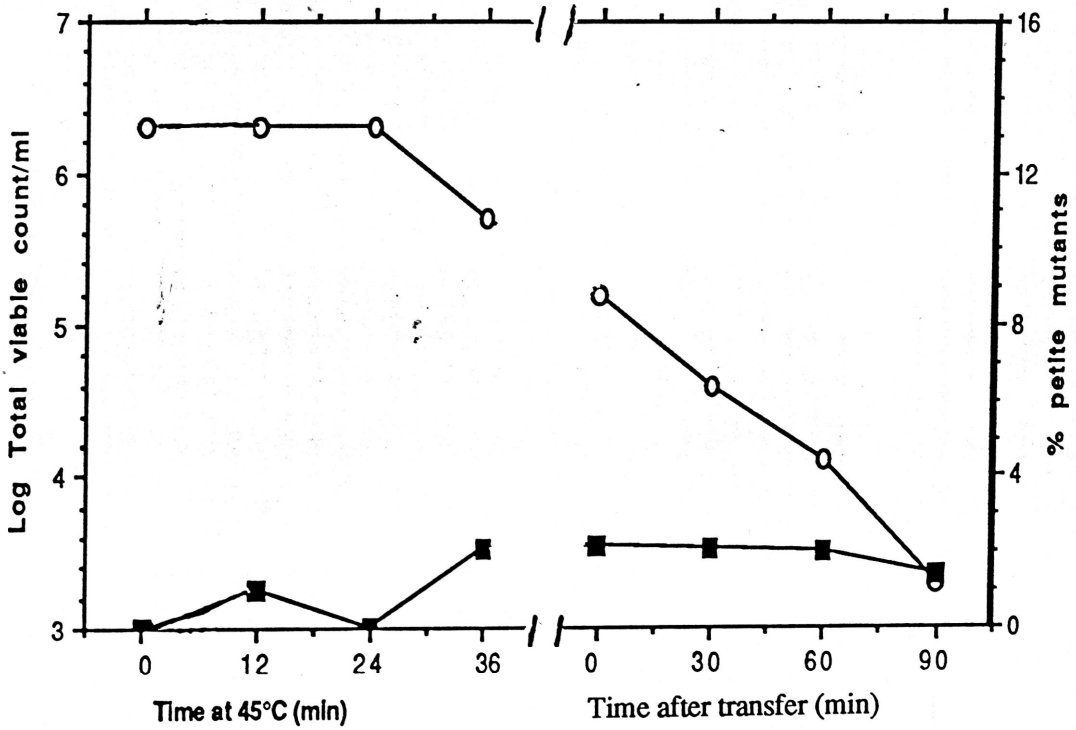


Figure 3.9 Effects of pre-conditioning at 45°C on viability (○) and proportion of *petite* colonies (% of total viable count) (■) of *Saccharomyces cerevisiae* strain Y41 response to BYM at 52°C. The second stage mid-exponential phase cells grown at 28°C were incubated for 36 mins (see MATERIALS & METHODS) 45°C before 10% v/v was used to inoculate the experimental flasks containing similar medium already equilibrated to 52°C.
Petites were identified by colony sizes.

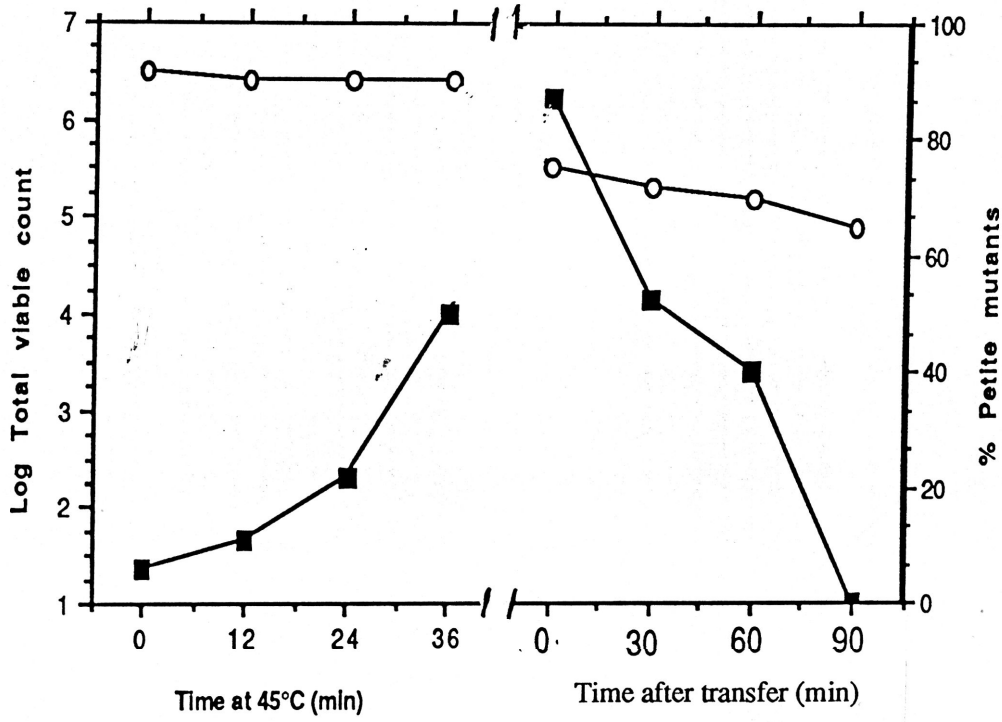


Figure 3.10 Effects of pre-conditioning at 45°C on viability (○) and proportion of *petite* colonies (% of total viable count) (■) of *Saccharomyces cerevisiae* strain 211-244-1A (*glc* 1) response to BYM at 52°C. The second stage mid-exponential phase cells grown at 28°C were incubated for 36 mins (see MATERIALS & METHODS) at 45°C before 10% v/v was used to inoculate the experimental flasks containing similar medium already equilibrated to 52°C.
Petites were identified by colony sizes.

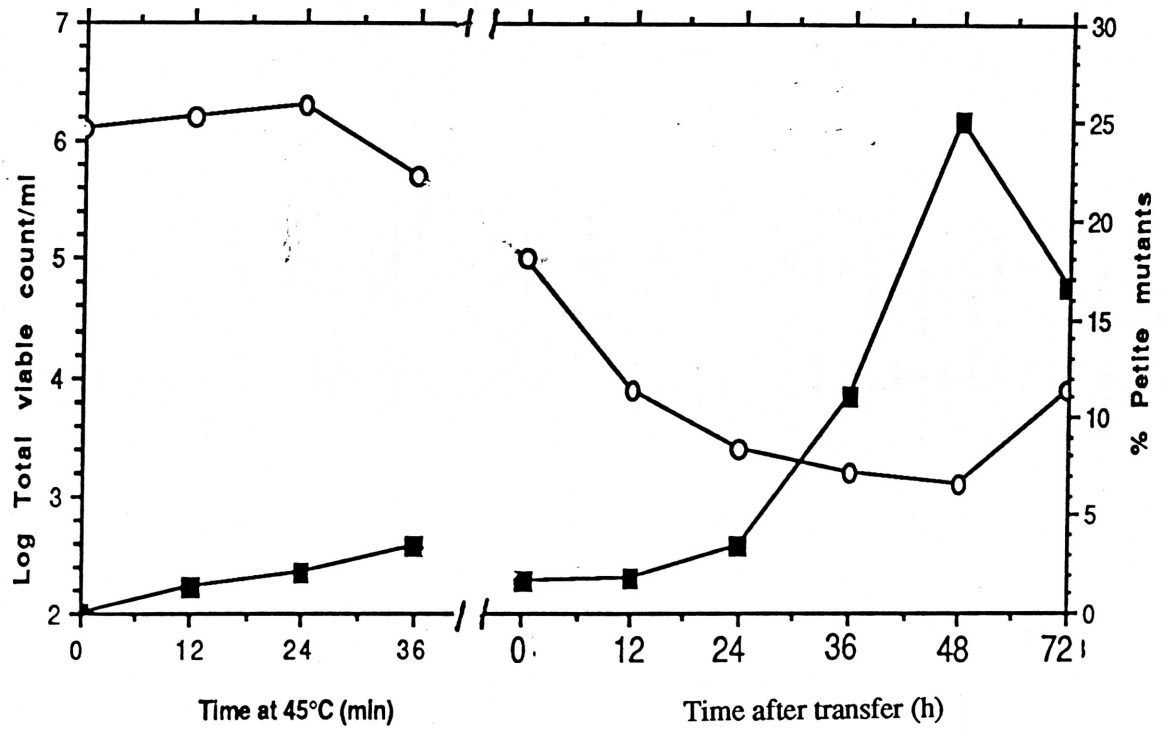


Figure 3.11 Effects of 45°C preconditioning on salt stress induced *petite* mutagenesis (■) and viability (O) of *S. cerevisiae* strain Y41. The second stage mid-exponential phase cells, grown at 28°C, were incubated for 36 mins at 45°C before 10% v/v was used to inoculate the experimental flasks containing salt broth already equilibrated at 30°C. Petites were identified by colony sizes.

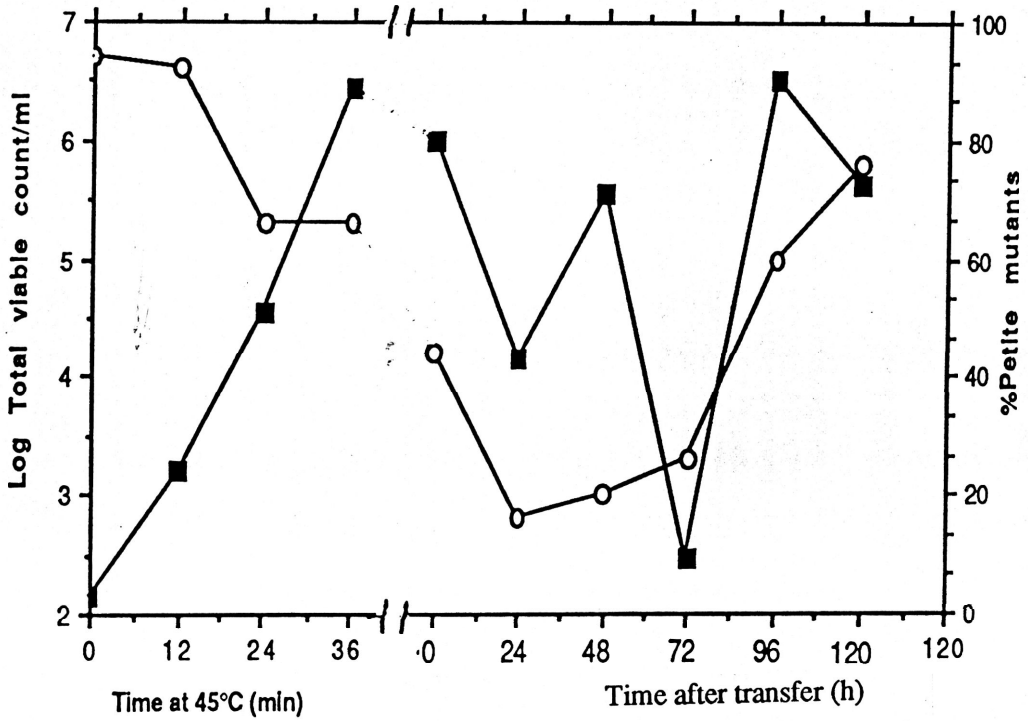


Figure 3.12 Effects of 45°C preconditioning on salt stress induced *petite* mutagenesis (■) and viability (○) of *S. cerevisiae* strain 211-244-1A (*glc* 1). The second stage mid-exponential phase cells, grown at 28°C, were incubated for 36 mins at 45°C before 10% v/v was used to inoculate the experimental flasks containing salt broth already equilibrated at 30°C.
Petites were identified by colony sizes.

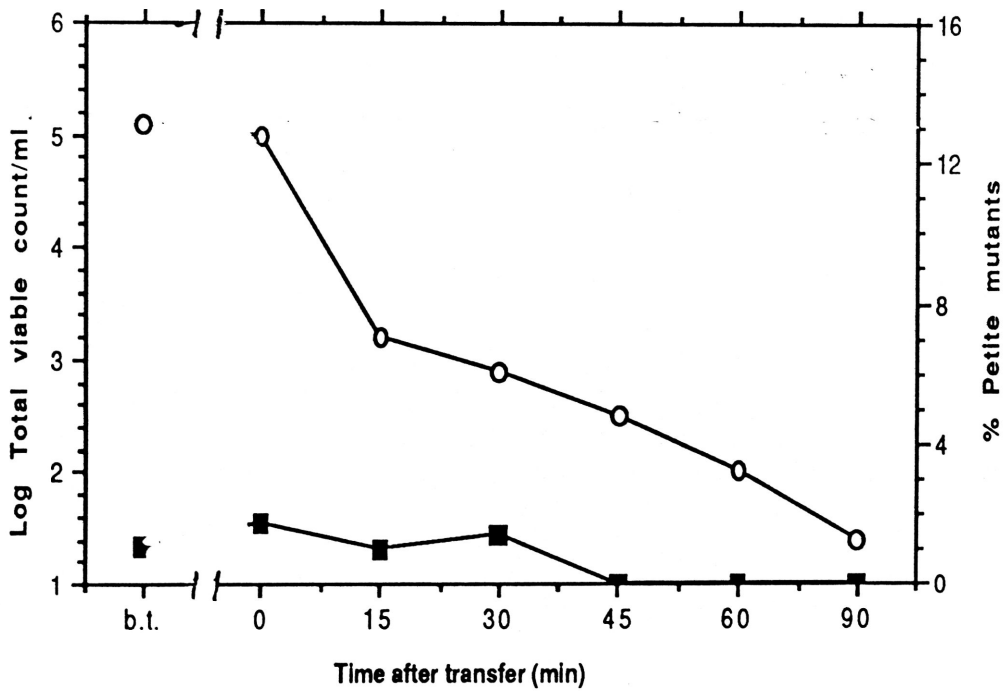


Figure 3.13 Effects of pre-conditioning in 2% (m/v) NaCl on viability (○) and proportion of *petite* colonies (% of total viable count) (■) of *Saccharomyces cerevisiae* strain Y41 at 52°C. A 1% v/v proportion of the first stage culture was used to inoculate second stage flasks containing 2% (m/v) NaCl. This was grown to mid-exponential phase before 10% v/v was used to inoculate experimental flasks containing BYM already equilibrated at 52°C.
Petites were identified by colony sizes.

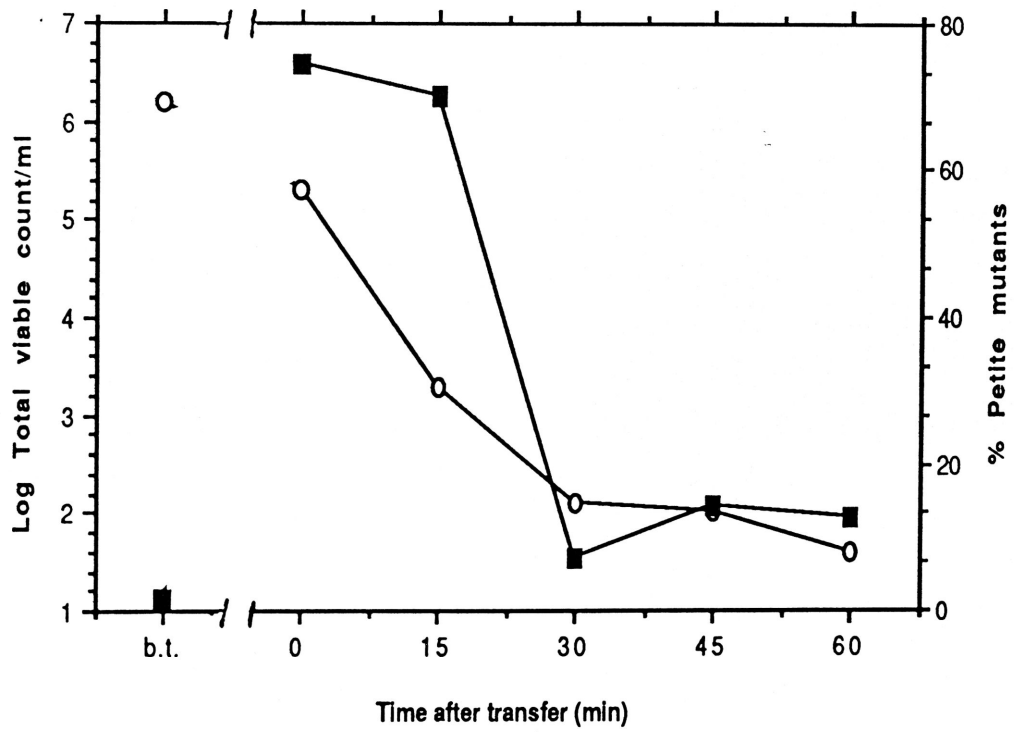


Figure 3.14 Effects of pre-conditioning in 2% (m/v) NaCl on viability (○) and proportion of *petite* colonies (% of total viable count) (■) of *Saccharomyces cerevisiae* strain 211-244-1A (*glc* 1) at 52°C. A 1% v/v proportion of the first stage culture was used to inoculate second stage flasks containing 2% (m/v) NaCl. This was grown to mid-exponential phase before 10% v/v was used to inoculate experimental flasks containing BYM already equilibrated at 52°C
Petites were identified by colony sizes.

3.4 TREHALOSE ACCUMULATION

Since trehalose had been reported to play some role as a compatible solute (see Brown 1978; Edgley & Brown 1983; Mackenzie et al. 1986), and also a possible protector of yeasts against heat and dessication (Attfield 1987; Hottiger et al. 1987), it was considered that the protective effects of preconditioning and incubation at 45°C for 36 mins before being used in the experimental stage cultures might have been associated with accumulation of trehalose. Changes in trehalose content during the 45°C preconditioning period were therefore determined. During this period, trehalose accumulated progressively to a level of 18.7 and 4.1 mg. g^{-1} (dry mass of yeast) respectively within strains Y41 (Fig. 3.15) and *glc 1* (Fig. 3.16).

Furthermore, as seen in Figures 3.15 and 3.16 the wild type contained more intracellular glucose concentration than the *glc 1* strain. Glucose content declined progressively in Y41with exposure to 45°C, but increased in *glc* 1.

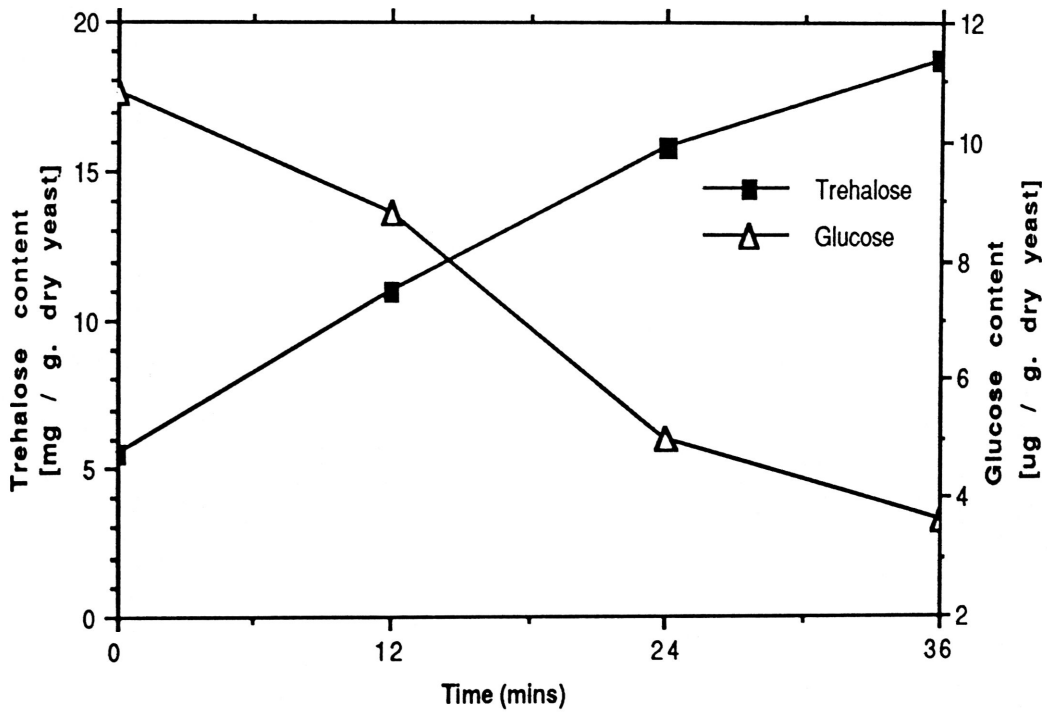


Figure 3.15 **Effects of 45°C preconditioning on the intracellular content of the dissaccharide trehalose and monosaccharide glucose of the wild type *Saccharomyces cerevisiae* strain Y41. Four separate flasks containing 100 ml BYM second stage culture growing to mid-exponential phase before exposure to 45°C temperature were used for each sampling time.**

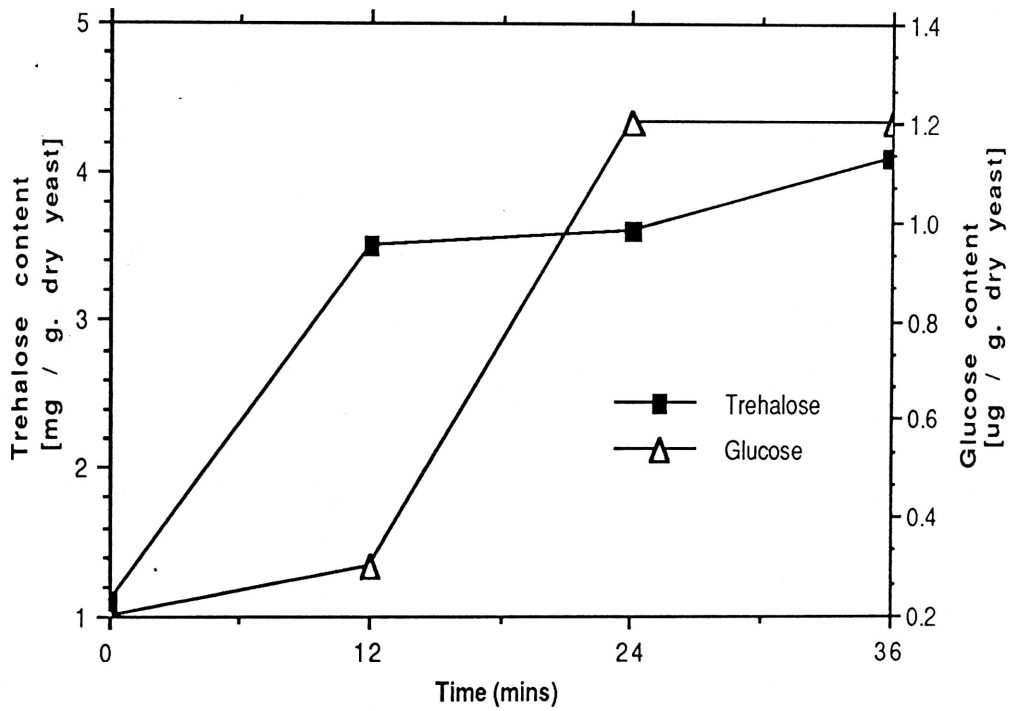


Figure 3.16 Effects of 45°C preconditioning on the intracellular content of the dissaccharide trehalose and monosaccharide glucose of the *Saccharomyces cerevisiae* strain 211-244-1A (*glc* 1). Four separate flasks containing 100 ml BYM second stage culture growing to mid-exponential phase before exposure to 45°C temperature were used for each sampling time.

CHAPTER FOUR : DISCUSSION

4.1 THE EFFECT OF SALT STRESS ON THE VIABILITY OF *SACCHAROMYCES CEREVISIAE* AND THE PRODUCTION OF *PETITE* MUTANTS

A previous study in this laboratory examined the physiological response when mid-exponential stage two cultures of *Saccharomyces cerevisiae* were transferred into experimental flasks containing salt broth (Edgley and Brown 1983). Edgley and Brown (1983) investigated the changes in viable count of this yeast by plating cultures on both a conventional high (MA) and low (SHA and salt agar) a_w agar media. As described in Section 1.5, the current project was prompted by the studies of Edgley and Brown (1983). However, its aim was to investigate the proportion of *petite* mutants arising during transfer experiments of this sort (Petelo 1985).

Before discussing the effect of salt stress on viability and the proportion of *petite* colonies, it is noteworthy to look at some interpretations of the results of Edgley and Brown (1983). There were two stages of adaptation of *S. cerevisiae* mid-exponential phase cells to the salt broth. Stage 1 was marked by a catastrophic drop of the apparent viability and different plate counts. Stage 2 was characterized by a period of recovery from stress, as delineated by an increase in viability to the size of the pre-transfer inoculum and accompanied by similar plate counts. The interpretation of the above results is not as straight forward as it might first seem (Brown 1990). For instance, in stage one the yeast cells are subjected to a dilution stress when plating onto MA. It is not clear however, whether the 'cell death' in stage one occurred in the salt broth, transfer to or after incubation of plate cultures, or during serial dilution (see Edgley and Brown 1983). Another question is whether the general recovery in stage 2 arose from a residue of survivors or from the majority of the yeast cells in stage one which was not 'killed' but had merely become dormant and unable to proliferate on the plating media.

Studies by Mackenzie et al. (1986) addressed some aspects of these interpretations. In this particular investigation, they found what is called 'water stress plating hypersensitivity'; a phenomenon that occurs when *S. cerevisiae* progressing in a normal growth cycle is plated onto low (SHA or salt agar) and high (MA) a_w agars. Consequently, a large plating discrepancy occurred when *S. cerevisiae* cells reached mid-exponential phase. Under most conditions, the discrepancy (log MA count - log

stressing agar count) was at least about 4 which means one cell in 10^4 survived and muliplied on stressing agar. The discrepancy diminished as the growth cycle progresses to stationary phase. Addition of either glycerol (0.5 M) or betaine (0.5 mM) to the low water potential (a_w) agar (s) had no effect on the magnitude of the discrepancy. The magnitude of the plating discrepancy is a function of the solute concentrations (or a_w) of the stressing agar media, which is roughly proportional to the solute concentration (a_w) at least above a critical threshold. Below that threshold value, there is no discrepancy (Mackenzie et al. (1986). Mackenzie et al. (1986) also tested some colonies from SHA which were plated during mid-exponential phase of the growth cycle to examine if they were resistant to stress. They found that these colonies and the original culture were equally hypersensitive. They concluded that failure of *S. cerevisiae* to grow in stressing agars was the result of death, not dormancy. In addition, the heterogeneity of the yeast population was physiological, not genetic. Therefore, it is likely that the recovery showed in stage two arose from residue of survivors rather than the whole population.

The phenomenon found by Edgley and Brown (1983) has been reproduced by Petelo (1985), Mackenzie (1988), and also in the present study. Reduction in viable count was observed in three strains of *S. cerevisiae,* 182-6-3 (cdc 24) (Figure 3.1a), Y41 (Figure 3.1b), and 211-244-1A (*glc* 1) (Figure 3.1c) after transfer of stage two mid-exponential phase cultures into salt broth. The recovery period of viability was apparent in strains 182-6-3 and 211-244-1A under incubation times used. A slight recovery of viability ('stage 2') for Y41 at 48 h was observed. All viable counts were determined on MA.

The resistance of the stationary phase cells adaptation to salt broth (see also Section 4.2) as compared to mid-exponential phase cells has made the interpretation of these results more easier. Mid-exponential phase cells are more sensitive to salt stress than stationary phase cells. Second stage stationary phase culture of *S. cerevisae* strain 182-6-3 (cdc 24) continued to grow in salt broth after a lag phase (Section 3.3). In addition, the mutation rate was steady and reflected the 'spontaneous' proportion of the mutants that arose in 'pre-stress' period. A similar characteristic was found in stationary phase cells of strain Y41 when transferred to the salt broth (Petelo 1985). This resistance of *S. cerevisiae* stationary phase cells to salt broth may be similar to the response of the mid-exponential cells of *S. rouxii* (Edgley and Brown 1983)

and *Debaromyces hansenii* (Larsson and Gustafsson 1987) although they tend to accumulate different compatible solutes. Moreover, mid-exponential cultures are generally sensitive to stress (or low a_W). The sensitivity of exponential phase culture to low a_W has been tested in 12 yeast species from 5 genera (Mackenzie 1988). Four (4) out of twelve (12) displayed water stress plating hypersensitivity. Two of which were strains of *S. cerevisae* while the other two sensitive yeasts were species of *Klockera* and *Candida krusei* . Notable among those yeasts that did not display sensitivity to diminish a_W is *S. rouxii*. . The viability of this yeast on MA and SHA was almost identical throughout the growth phases of the batch culture. In essence, Mackenzie (1988) showed that a sharp distinction between the resistant species and the sensitive strains was the presence of more than one polyol in the former but not the latter (see Section 1.3.3). The most notable polyol is glycerol. Glycerol is by and large the most well known polyol possessed by most eukaryotic microorganisms and functions as a compatible solute (protective) or an osmoregulatory solute (to maintain turgor), or both (Brown et al. 1986).

Glycerol is predominant in the exponential phase of the growth cycle and in most cases, it is responsible for maintenance of turgor (see Sections 1.3.2 & 1.3.3) especially when cells are subjected to water stress (see for example Brown 1990). When the growth cycle reaches stationary phase and the amount of glycerol is low, other polyols are accumulated by yeast cells. Xerotolerant yeasts such as *S. rouxii* (Brown 1978a) and *Debaromyces hansenii* (Gustafson and Nokrans 1976) accumulated more arabitol content than glycerol during stationary phase. This may help to explain the resistance of stationary phase cells of *S. rouxii* and *D. hansenii* . However, this may not be the case for *S. cerevisiae* because no polyol other than glycerol was detected in this yeast (Mackenzie 1988). As *S. cerevisiae* progresses to stationary phase, the dominant 'compatible solute' found to be accumulated by yeast cells is trehalose (see Mackenzie et al. 1988). These researchers have demonstrated that *S. cerevisiae* accumulated trehalose up to 50 mg (g dry yeast $^{-1}$) compared to a minimal amount of glycerol of about 2 mg (g dry yeast $^{-1}$). In essence, accumulation of trehalose in a *S. cerevisiae* mutant strain 211-244-1A (*glc* 1) was low and recovery of stress resistance was only slight. Hence, they concluded that the development of resistance in *S. cerevisiae* to water stress may have accompanied the accumulation of trehalose.

The discussion of the various means of reducing the catastrophic drop in viability after mid-exponential phase cells transfer to salt broth will be treated in the next Section (Section 4.2). Up until now, as discussed, most of the questions regarding adaptation to salt broth pertinent to this project have been answered. However, the intriguing part stemming from these early comparative studies (Edgley and Brown 1983) was the observation that a simple physicochemical stress such as salt stress imposed change in colonial morphology of the non-tolerant strain *S. cerevisiae* Y41 (see SECTION 1.5).

Transfer of mid-exponential cultures of *S. cerevisiae* strain Y41 to salt broth (a_W 0. 935) is accompanied by a massive drop of viability (stage 1) and a subsequent recovery to the pre-transfer level of the inoculum (Petelo 1985; see also Fig. 3.1a & Fig. 3.1b). These changes are associated with an increase in the proportion of *petite* mutants from 1 - 2 % (spontaneous level) before stress to a maximum of about 35 % after 72 h (Stage 2) (see also TABLE 3.1) in the salt broth; the mutants were viable only on MA. The prelimimary task of this project was to check whether this phenomenon of *petite* mutation occurred within other strains of *S. cerevisiae* and, to test whether this high proportion of *petite* colonies tested by streaking on MA (see MATERIALS & METHODS) is reproducible by using other testing criteria such as TTC and size measurement under stereo-microscope. Using these various testing criteria, *petite* mutants were observed in three strains of *S. cerevisiae* studied in this project, a temperature sensitive strain 182-6-3 (cdc 24) (Figure 3. 2a), a glycogen-deficient mutant strain 211-244-1A harbouring *glc* 1 mutation (Figure 3.2c), and a wild type strain Y41 (Figure 3.2b). In particular, the increase in the proportion of the mutants at 24 h after transfer to salt broth was more pronounced in strains Y41 and 211-244-1A than for strain 182-6-3. Table 3.1 shows a statistical analysis of the proportion of the mutants which is qualitatively similar to that shown by data on Figures 3.2. There are different rates of mutation for each strain. The mechanism of this salt-induced *petite* mutagenesis will be discussed in Section 4.3. However, we can conclude for now that the adaptation of *S. cerevisiae* mid-exponential phase cells to salt broth (see above) was accompanied by an increased proportion of *petite* mutants. This was found in Y41, a temperature-sensitive (t.s.) mutant cdc 24 and a glycogen-deficient mutant strain 211-244-1A. In addition, stationary phase cells of the t.s. strain were resistant to salt stress.

4.2 FACTORS AFFECTING VIABILITY AND THE PROPORTION OF *PETITE* MUTANTS ARISING AFTER TRANSFER EITHER TO A SALT STRESS OR A TEMPERATURE STRESS

Agents that induce *petite* mutation have been extensively documented, from chemical mutagens such as ethidium bromide to inhibitors of mitochondrial macromolecular synthesis such as erythromycin. In addition, *petite* mutation is also induced at high temperature, by nutritional and other related treatments (see Table 1.1 and Section 1.1). This induction process could be blocked, however, in some cases by various treatments and antagonists (see Section 1.1.2). One example of a treatment that blocks *petite* mutation is the use of an inhibitor of DNA synthesis such as sodium nalixidate. Whittaker et al. (1972) found that induction of *petite* mutants by ethidium bromide or 5-fluorouracil can be blocked by addition of sodium nalixidate. Although *petite* mutation can be suppressed by various inhibitors of DNA and other related macromolecules, not all can perform the same task; some inhibitors of macromolecular synthesis are themselves *petite* inducers (see Table 1.1 in Section 1.1).

This thesis reports some factors that affect the proportion of *petite* mutants (either by inducing or suppressing) arising when stage two mid-exponential phase cells were subjected either to a salt stress or a temperature stress. They are: age of culture, temperature, and preconditioning (either by 2% NaCl or 45°C preconditioning prior to transfer).

As mentioned in Section 3.3.1, the production of *petite* mutants diminishes as the yeast culture ages (Schenberg-Frascino and Moustacchi 1972; Wallis and Whittaker 1974; Petelo 1985). Stationary phase cells of *S. cerevisiae* have also been known to be resistant to 52°C heatshock (Schenberg-Frascino and Moustacchi 1972; Parry et al. 1976; Walton et al. 1979). Table 3.3 shows the resistance of stage two stationary phase cultures of *S. cerevisiae* t.s. strain cdc 24 transfer to experimental flasks containing salt broth. The proportion of *petite* mutants that arose in the salt broth reflected the mutation rate at the 'spontaneous' level (cc. to 'before stress' level in

Figure 3.2a or in Table 3.1; see Section 1.1.3).

The transfer of mid-exponential phase cultures of strain 182-6-3 (cdc-24) to salt broth at 20°C did not result in a low proportion of *petite* mutants, although the apparent viability dropped by about 1000-fold (Figure 3.5). In addition, the effect of plating into two different temperatures was not significantly different (20°C and 30°C, see Fig. 4.5). Yanagashima (1967) incubated *S. cerevisiae* var. *ellipisoideus* at 4°C in medium containing salts. He found that *petite* mutation was not induced, even with 2 days incubation and concluded that metabolic activity is important for induction of respiratory deficiency. On the other hand, when stage two mid-exponential phase cultures of Y41 were transferred to salt broth at 20°C, the proportion of the mutants was always low. TTC (2,3,5-triphenyl tetrazolium chloride) was used to test *petites* on both cultures of Y41 (result not shown), and cdc 24 (Figure 3.5) for experiments at 20°C. The paradox may be due to strain difference. There was no further work to establish their significance.

Furthermore, the responses of the mid-exponential phase cultures were quite different at 52°C (Fig. 3.6 & 3.8) from those at 30°C (Table 4.1). In both cases, there was a decrease in viability with (30°C) or without salts (at 52°C). However, it seems that *petites* may have higher death rate than the normal colonies at high temperature (Fig. 3.6 and 3.8). Note that the decline in the proportion of *petite* at 52°C may be attributed to higher death rate of the *petite* cells. In NaCl at 30°C , however, the decline in the proportion of the mutants could be the result of a decreased mutation rate and increased growth rate of the parent strains relative to mutation rate. This was apparent in strain 211-244-1A in both preconditioned and non-preconditioned cultures. In both conditions, the mutation rate gradually diminished after long incubation in the salt stress. In general, however, these results may suggest that yeast cells have a higher death rate in at high temperature than when in salt broth. At both 52°C and 30°C, the *petite* cells are killed off faster than the normal cells. There was clear evidence of protection by salt against the lethal effects of the high temperature. Both the viability and the proportion of *petite* mutants were partially protected (Figure 3.7; see also Wilson et al. 1978). This indicates that there is an opposing effect of high temperature and high NaCl concentration on both death rate and proportion of mutants. Specifically at 30 mins, the viability was increased by about 10% compared to 52°C experimental cultures without salt (Fig. 3.6).

The opposing effect of high salt concentrations and high temperature has been shown in a wider range of biological processes. For example, media containing sucrose or sodium chloride increase the heat resistance of osmophilic yeasts (Gibson 1973; Corry 1976) and vegetative cells of *Saccharomyces bailii* and *S. cerevisiae* (Wilson et al. 1978). In addition, an increase in temperature increases the sugar concentration that a yeast could tolerate, and decreases the optimum a_w for growth (Ingram 1957). This opposing effect could affect some of the sensitive reactions in cell metabolism. One of these sensitive reactions is the level of transcription. In a 1 *gal* DNA transcription system, stimulation of RNA synthesis has been shown to be proportional to glycerol concentration in vitro (Nakanishi et al. 1974), and the effect is temperature dependent. Glycerol also overcame a requirement of cAMP for the promotion of λ *gal* RNA with a transcription system using λ *gal* DNA and *Escherichia coli* RNA polymerase. These authors suggested that glycerol may act by changing the conformation of the DNA template rather than by affecting the properties of RNA polymerase. They pointed out that glycerol and salt have opposite effects on DNA stability; glycerol lowers the DNA melting temperature (T_m value) whereas salt increases it. Other solutes that stimulate *gal* and total RNA synthesis are sucrose, ethylene glycol, dimethylsulfoxide, and 1, 3-propanediol. All of these solutes lower the DNA melting temperature. In addition, glycerol, ethylene glycol, glucose or sucrose have been shown to increase/ stimulate "transcriptive activity of crude or partially purified rat thymic RNA polymerase 11 on calf thymus DNA" (Buss and Stalter 19 78). They also reported that transcription is dependent of concentration and is pronounced as low as 1-2 % (v/v). On a molar basis, transcription stimulation bears relation to the number of hydroxyl substituents (sucrose ≥ glucose ≥ glycerol). As in the prokaryotic system above, the glycerol mediated trancription is temperature dependent. Moreover, stimulation of RNA polymerase by 25% (v/v) glycerol was less effective at 23°C than at 37°C. In addition, it was more pronounced at 46°C than at 37°C.

Moreover, the level of enzyme activity is protected by glycerol even in high glycerol concentration. Glucose-6-phosphate dehydrogenase activity from *Dunaliella* was not inhibited by action of glycerol at up to 4M, in contrast with the inhibitory effects of salt at substantially lower concentrations (0.4M) (Borowitzka and Brown 1974). In addition, salt-tolerant higher plants (halophytes) accumulate glycine betaine (Storey and Wyn Jones 1977) or proline (Stewart and Lee 1974; Stewart and Hanson 1980)

when subjected to salt stress, whereas a diverse range of marine animals accumulate various amino acids and quaternary ammonium compounds in response to osmotic stress (Yancey et al. 1982). In all cases where enzymes were isolated from organisms growing in saline media, NaCl has proved to be more inhibitory than the dominant intracellular organic solute (Yancey et al. 1982; Wyn Jones and Gorham 1983). All these accumulated solutes are behaving as 'compatible solutes' like glycerol in yeast.

In light of the above findings, it can be envisaged that the same effects may occur in yeast. Accumulations of intracellular glycerol may promote activation of specific promoters of RNA synthesis. On the other hand, yeasts are known to possess a Na^+ extrusion mechanism which pumps out most of the Na^+ to the medium in case of high salinities or high salt concentrations. Thus, the opposing effect of salt and high temperature may create the mechanism that suppresses catastrophic reduction in viability (see Figure 3.7). Since exposure to salt stress causes accumulation of compatible solute (s) in yeast and other microorganisms, and transfer to 52°C triggers the formation of hsps, there is presumptive assumption that these are the factors that may help protect viability. Hence we may suggest that a formation of *petites* , which is partially suppressed in salt broth at 52°C, may be due to cells that fail to accumulate compatible solute such as glycerol and other related polyols. This may lead to defective promoters of the transcription system.

Although salt stress or temperature stress result in changes of viability and proportion of *petite* mutants, these effects can be partially suppressed by preconditioning. Preconditioning in 2% NaCl had different effects on the proportion of *petite* mutants produced by a larger salt stress (10% NaCl) in the three strains of yeast. Strain 182-6-3 (cdc 24) had a higher proportion of *petites* than 'normal' at the end of 2% NaCl preconditioning which may be attributed to mild stress incurred by 2% NaCl (Table 3.4). This proportion did not increase on transfer to 10% NaCl. Mutation rate was almost similar during stress phase (Table 3.4). In addition, the maximal proportion of salt stress-induced *petites* was apparently diminished in strains Y41 and 211-244-1A (*glc* 1). However, in strain 211-244-1A the temporal pattern was affected. There was a lower proportion of *petites* in *glc* 1 later in the incubation period. The improved survival of all strains may be attributed to glycerol accumulation (c.f. Mackenzie et al. 1986). However, the role of glycerol in the improvement of survival of cells that had been preconditioned at 2% NaCl can be questioned. Recent study by Blomberg and Adler (1989) found that removal of

glycerol from cells that had been preconditioned caused little change of their shock tolerance. In essence, they considered that enzyme glycerol phosphate dehydrogenase (GPDH), which links glycerol formation to glycolysis, as well as other proteins might be coordinately regulated in order to express power as a determinant in the osmotolerance phenotype of the cells. Moreover, the effect on the proportion of *petite* accumulation during incubation in salt stress in Y41 is probably more complex and might reflect secondary effects in a population with a higher proportion of viable cells. In strain 211-244-1A, the decrease in mutation rate later in the salt broth may be due to death of *petite* cells. Again, it may be due to an effect on a sensitive reaction such as DNA-directed RNA polymerase (transcription) (see above). In addition, it seems that prolonged incubation with salt stress during stage 2 (recovery period) may lead to a general decrease in mutation rate in both preconditioned and non-preconditioned cultures. This effect is quicker in the preconditioned cultures (see Table 3.4). Hence, we can conclude that 2% NaCl preconditioning suppresses *petite* mutagenesis at some stages during adaptation to the salt broth for Y41, 182-6-3 (cdc 24) and 211-244-1A. In addition, this preconditioning enhances viability in all *S. cereviae* strains above.

Moreover, 2% NaCl preconditioning partially suppressed *petite* mutation and viability in mid-exponential cultures of Y41 when transferred to a BYM at 52°C (Fig 3.13). The effect was less in strain 211-244-1A (*glc* 1) (Figure 3.14). Specifically at 30 mins at 52°C (Figure 3.14), there was an approximate 10 times increase in viability of strain 211-244-1A 2%NaCl preconditioned cultures compared to cultures that had not been preconditioned (Figure 3.8). The results with strain 211-244-1A (a mutant harbouring *glc* 1 mutation which is defective in glycogen and trehalose) suggest that trehalose might also be involved. This preconditioning has a delayed effect most notably demonstrated by *glc* 1 (Table 3.4), suggesting that its effect is indirect affecting the dynamics and course of adaptation and growth; and indirectly through the proportion of mutants.

Pretreatment at 45°C protects against lethal effect of salt stress, but does not seem to suppress mutation rate. In addition, the results suggested that trehalose may not be involved in suppressing *petite* mutagenesis under those circumstances. There was an apparent oscillation of proportion of *petites* of *glc* 1 (Fig. 3.12) when a preconditioned culture was exposed to salt broth. On the other hand, 45°C pretreatment protects against the lethal effects of high temperature, and , it seems to

suppress the apparent mutation rate (Fig. 3.9). The effects of the preconditioning of strains Y41 and *glc* 1 clearly suggest that the formation of *petites* in a temperature stress is related to the content of trehalose. Y41 with a relatively high content of trehalose than *glc* 1 during the preincubation period at 45°C had a low proportion of *petite* mutants than the latter (Figures 3.15 & 3.16).

Furthermore, the incidence of *petite* mutants in *glc* 1 was enhanced greatly by either 45°C (preconditioned temperature) or 52°C, and continued incubation decreased it. The *petites* of strain 211-244-1A are presumably sensitive because they seem to be killed faster than the *grandes* . In addition, quantitative measurement of the trehalose content in the glycogen-deficient mutant showed an increased level of tehalose accumulation during 45°C preconditioning period (Figure 3.16), although this amount was lower than the amount accumulated by the wild type strain Y41 (Figure 3.15). This finding does not rule out some involvement of trehalose as a protectant. Since *petite* colonies are known to lack many respiratory enzymes in yeast mitochondria (see Section 1.1), it is possible that this high proportion of *petite* mutants at 52°C may be due to *petite* cells that are defective in various pathways leading to trehalose synthesis. Panek and Matoon (1977) found a correlation in the activity of the trehalose accumulation system and the concentrations of cytochromes when studying the effects of oxygen limitation. These authors suggested that trehalose accumulation is directly dependent upon the activity of mitochondria in producing ATP. Although no quantitative measurement of the trehalose from the *petite* mutants arising at 52°C was carried out, it is not unwise to suggest that the high death rate of *petite* cells at 52°C may be due to a failure of *petites* to accumulate sufficient trehalose or other protective agents (e.g. hsps) which is caused by defects induced by high temperature on precursors of trehalose synthesis (see Section 4.3).

In general, the results in this project suggest that both 2% NaCl (Glycerol effect) and 45°C (Trehalose effect) preconditioning play a role in attainment of growth of *S. cerevisiae* in response to either salt stress or temperature stress. It is possible that both compatible solutes are involved in this effect. Edgley and Brown (1983) reported the detection of trehalose at 20 h ('stage 1') onwards, when a mid-exponential culture of *S. cerevisiae* was adapted to salt broth, although no quantitative measurement of the dissaccharide was done. Mackenzie et al. (1988) showed that the development of resistance to plating discrepancy occurring during

late mid-exponential phase and was accompanied by an increase level of trehalose accumulation. This resistance was apparently unrelated to glycerol content which was always low. In essence, when the trehalose content reached at least about 50 mg (g dry yeast)$^{-1}$, the yeast were fully resistant to stress imposed by low a_w . They concluded that trehalose was a more effective compatible solute , per mole, than glycerol. In the current study, the results for the accumulation of trehalose at 45°C, the wildtype accumulating about 4.5 times as much disaccharide as *glc* 1 (Figures 4.15 and 4.16) suggest that trehalose might have played a role in protecting the viability; failure of the yeast cells to accumulate trehalose may cause an induction of *petite* mutants by temperature stress. The possibility that heat shock proteins are involved as protector against stress, thus helping cells to recover, cannot be overlooked (see Section 1.4. 2).

The heat shock system affecting dynamics of the relationship between the *Drosophila melanogaster* genome and one of its retrotransposons, copia, has been studied by Strand (1988). He found that an insertion of a copia element into the *adh* gene reduces the *adh* transcript abundance and disrupted normal developmental promoter usage. The mechanism by which this transposon insertion altered *adh* expression involves not only displacement of cis-acting controlling elements, but also interference by copia element expression. Since transposable elements are also found in yeast, and can be a major source of mutation, it is possible to suggest that 45°C preincubation induces production of heat shock proteins together with trehalose accumulation (Attfield 1987; Hottiger et al. 1987) indirectly protecting any source of mutation, either by a transposable element, heat or salt stress. The effects of heatshock proteins and trehalose may be an indirect one through the mitochondrial DNA. It is also possible that glycerol is involved; thus, suggesting that compatible solutes and heat shock proteins share the role as protector against stress or heat stress. The protective nature of glycerol in heat stress must be of compatible solute nature (see Section 4.3).

4.3 POSSIBLE MECHANISM OF PETITE MUTATION UNDER SALT STRESS

As described in Section 4.1, the catastrophic decrease ('stage 1') in the apparent viability of a mid-exponential phase culture transfer of *S. cerevisiae* to salt broth (BYM containing 10% NaCl) and its subsequent recovery to pre-transfer level ('stage 2') of the inoculum was associated with a massive increase in the proportion of *petite* mutants of up to 35 % at 72 h (in 'stage 2') (Petelo 1985). Approximately 25% of the *petite* colonies isolated were stable (i.e. producing unchanged size of *petite* colonies after streaking and incubated for 2 days) whereas, about 10% were unstable or revertible, producing two types of colonies, *petite* and *grandes* , after streaking and incubation for 2 days.

The revertible *petites* reflect the heterogeneity of the *petite* population (Petelo 1985), and perhaps the "degree of suppressivity" (Ephrussi et al. 1955). Ephrussi and associates suggested that the suppressive nature of *petites* implies that a mutation is an overall consequence of loss of a nonchromosomal genetic determinant. It was suggested that these two categories of *petites* may be largely of the neutral type (p^0) for stable mutants, and suppressive (p^-) for revertible *petites* (Petelo 1985). Constanzo and Fox, unpublished results cited in Fox (1986), have also reported a respiratory deficient mutant (*pet* 494) that is revertible resulting in production of *grandes* (*rho* $^+$) and petites (*pet* 494; *rho* $^-$). The term, *pet* , signifies *petite* colonies that undergo nuclear mutations and subsequently leads to respiratory deficiency (Chen et al. 1950; Sherman 1963; Sherman and Slonimski 1964). Nuclear mutations of this type have been found to block the expression of specific mitochondrial genes post-transcriptionally (Fox 1986). Constanzo and Fox (unpublished results cited in Fox 1986) also found that *pet* 494 mutant cells are capable of accumulating cox 111 (i.e. the subunit 3 of the respiratory enzyme cytochrome oxidase) encoded by rearranged genes (genes which are carried on *rho* $^-$ mtDNA molecules of the kind normally found in other *petite* strains), and thus they respire and grow on non-fermentable carbon source such as glycerol despite the nuclear *pet* 494 mutation.

Although there was no genetic test carried out to establish the significance of the unstable *petites* (Petelo 1985), it is possible, following the above report, that they belong in the category of *pet* mutants. Nevertheless, with the 25% of stable *petites*

(Petelo 1985), already a high proportion, it was observed that the maximum proportion of the mutants arose at 48 h and 72 h during 'stage 2' in the salt broth. Two questions arose; (1) was the increase of the proportion of *petite* mutants in salt broth a result of a greater resistance (differential selection) of the pre-existing *petite* strains to salt stress ? OR, (2) was it simply due to mutagenic action of the stress ?

Screening for death rates in the salt broth of *petite* strains isolated before/after stress against parental strain have shown, that this increased proportion of *petite* mutants in 'stage 2' was not a result of an enhanced survival of *petite* strains in salt broth and must therefore be a result of a mutagenic action of the salt stress (Fig. 3.3; Tables 3.2a & 3.2b). In addition, there were different patterns and rates of mutation among the three strains. Y41 showed a progressive increase in rate of *petite* mutation to 72 h. Cdc 24 showed a peak at 24 h and a relative plateau in *glc* 1 strain was apparent at 24 h to 72 h (Table 3.1). Despite these different patterns, all strains showed an increased proportion of *petite* mutants during exposure to salt stress. Moreover, stationary phase cultures of cdc 24 showed a constant proportion of *petite* mutants after transfer to salt broth. Similar results had been found for Y41 (Petelo 1985). This suppression which is shown by the rate of mutation in stationary phase cultures may be due to accumulation of trehalose (Panek and Matoon 1978) (see Sections 4.1 and 4.2).

Trehalose, which has been found to correlate with the development of resistance of yeast cells exposing to a low a_w medium (Mackenzie et al. 1988),may be responsible for the high level of *petite* colonies in 'stage2'. Table 3.1 shows a high proportion of *petite* mutants in 'stage 2' for strains Y41 ('stage 2' is after 24 h onward) and 211-244-1A ('stage 2' is after 48 h onward), but not for strain 1882-6-3 (cdc 24) (the temperature-sensitive strain). Experiments of Attfield (1987) and Hottiger et al. (1987) help to make this interpretation easier. Both laboratories have shown that trehalose is accumulated in *S. cerevisiae* during exposure to agents that induce heat shock response. In common, they both measured the content of trehalose when transferred from an optimal temperature (27 or 30°C) to an intermediate/ higher temperature. Consequently, trehalose content increased when exposed to intermediate/ higher temperature and subsequently decreased when returned to the optimal temperature range. Both groups concluded that trehalose and heat shock proteins appear to share the characteristic of functioning in normal cell cycle and development as well as in recovery from stress.

Moreover, the synthesis of heat shock proteins occurs in cells shifted to higher temperatures (Lindquist 1986). This response is appeared to be transient because the rate of hsps synthesis reaches a plateau after about 90 mins and subsequently decreases when cells are returned to normal growth temperatures (Lindquist 1986; MacAlister and Finkelstein 1980; Miller et al. 1982). Figure 3.11 and Figure 3.12 show the response of stage two mid-exponential cultures of *S. cerevisiae* strains Y41 and 211-244-1A preconditioned at 45°C before being transferred to salt broth media which were equilibrated at 30°C. Both strains showed an increase proportion of *petite* colonies during the preconditioning period and also at subsequent transfer into the salt broth media. Attfield (1987) found that the content of trehalose in heat shocked cells diminished rapidly when *S. cerevisiae* returned from 45°C to 30°C. He considered that the growing yeast cells do not accumulate trehalose under optimal growth condition and thus mobilise any existing deposit of the dissaccharide when growing on glucose under normal physiological conditions (see also Thevelein 1984). In addition, he suggested that the biosynthesis and breakdown of trehalose by trehalose-6-phosphate synthase and trehalase , respectively, is controlled by cyclic AMP-dependent protein phosphorylation. Therefore, trehalose-6-phosphate synthase is phosphorylated and inactive, whereas trehalase is phosphorylated and active, during normal growth on glucose when cyclic AMP levels are high (Panek et al. 1987; Francois et al. 1987; Wiemken and Schellenberg 1982). Given these findings the return of heat shocked culture to normal growth tremperature may be suitable to provide the correct physiological state for mobilization and rapid diminution of stored trehalose. Transferring 45°C preconditioned mid-exponential cultures to salt broth at 30°C only suppressed the reduction in viability but not *petite* mutagenesis. The effect is greater in strain 211-244-1A (*glc* 1). When these cultures were transferred to BYM at 52°C, *petite* mutagenesis was only suppressed in Y41 but not with *glc* 1 strain. Since *glc* 1 strain is defective in glycogen and trehalose metabolism (Ortizet et al. 1983) and considering the known effects of trehalose, it can be suggested that the mechanism (s) underlying the formation of *petite* colonies under temperature stress may involve trehalose (or other compatible solutes such as glycerol) and perhaps heat shock proteins.

The induction of the mutants under salt stress may be due to toxic effects of the Na^+ ions. Sodium chloride salt is a very toxic substance to microorganisms; in general, it affects the activity of enzymes. In order for an organism to survive under water stress

(or solute stress), its enzyme must be functional (Brown 1978a). A study using isocitrate dehydrogenase from *Halobacterium salinarium* demonstrated that KCl is a much less effective inhibitor of enzyme activity than NaCl (Aitken and Brown 1972). All halotolerant and halophilic microorganisms discriminate to some extent against external NaCl, thus maintaining lower intracellular Na^+ concentration by a combination of low plasma membrane Na^+ permeability and active Na^+ extrusion (Reed 1984). In yeasts, this mechanism has been found for example in halotolerant *D. hansenii* and nonxerotolerant *S. cerevisiae* (Nokrans and Kylin 1969). The halotolerant (salt tolerant) yeast, *D. hansenii,* accumulates both K^+ and glycerol (Gustaffson and Nokrans 1976). According to the 'compatible solute' theory, Schobert (1977) suggested that the 'water-like' hydroxyl groups of polyols (e.g. glycerol and mannitol) replace water molecules and therefore maintain a hydrophobically enforced water structure within cytoplasm under conditions of lowered cellular water potential. *Petite* cells under salt stress are usually smaller and apparently more sensitive to stress than *grandes* (normal). We can assume that these mutants are facing complicated problems in maintaining constant cell volume and turgor pressure in the face of low a_w (see Section 1.2.2). Massive numbers of 'shrunken' or bursting cells were observed under the microscope when *S. cerevisiae* mid-exponential cultures were transferred into the salt broth (Results not shown). Perhaps the injured yeast cells which survived 'phase 1' (see Brown 1976, 1978a and Section 1.2.2) are those that were converted to *petites* as a result of the osmotic stress caused by high concentration of Na^+. The susceptibility of the non-tolerant strain *S. cerevisiae* to high Na^+ concentration, resulted in salt-induced *petite* mutagenesis, and the reported failure of the tolerant yeast *S. rouxii* to form *petites* (Kreger-van Rij 1969 cited in Brown 1978a) may be attributed to the inefficiency of the Na^+-extrusion mechanism in the former but not the latter.

Moreover, accumulation of trehalose was inhibited by incubation of cells in the presence of either acridine orange (ACO) and ethidium bromide (EBR) (Attfield 1987)). Since the heat induced-trehalose storage requirement for RNA synthesis resembles the heat shock response which is stimulated at the level of transcription (Attfield 1987), and considering the well known characteristic of EBR as a potent *petite* mutagen (Section 1.1.5.3.3) which acts by modifying the superhelical nature of DNA (Slonimski et al. 1968), we can assume that an enhanced formation of *petites* when 45°C preconditioned cultures of *S. cerevisiae* were transferred to a salt stress may be due to failure of *petite* cells to accumulate trehalose due to its defective

mitochondria.

Trehalose content is higher in stationary phase of growth than in exponential phase (see Mackenzie et al. 1988). During yeast exposure to low a_w (or salt stress), the main solute accumulated is glycerol (Brown 1978a) which contributes to adjustment of turgor. Trehalose, a reserve carbohydrate, is only accumulated as a reserve when other solutes are exhausted and it serves a 'compatible solute' role rather than an osmoregulator one. As described in Section 4.2, the effect of trehalose as an agent to confer resistance is effective when its content reaches at least 50 mg (g dry yeast)$^{-1}$. The results in this thesis show that the wild type strain Y41 (Figure 4.15) accumulated trehalose to a maximum of 18.7 mg (g dry yeast) $^{-1}$ after 36 mins preconditioning at 45°C. Thus, this content is only 37.4 % of the minimum amount required for attainment of a minimum total resistance to a solute stress. Perhaps that is why it does not confer full protection on viability or reduce the mutation rate. Similar quantitative techniques were used to assesss trehalose content in this project and Mackenzie et al. (1988).

Moreover, since trehalose content is always higher in stationary phase (Mackenzie et al. 1988), it might help the recovery of the viability and influence the proportion of *petite* mutants when a stationary phase culture is transferred to salt stress (low a_w or solute stress) (see Table 3.3; Petelo 1985). Petelo (1985) showed that a stage two stationary phase culture transferred to salt broth had a proportion of *petite* mutants which is equivalent to that found in 'before transfer' samples ('spontaneous level'). It is possible that this reduction in the proportion of the mutants in stationary phase is due to accumulation of trehalose (see for example Attfield 1987). In addition, it is possible that other agents or mechanisms are involved. Before discussing further, the involvement of compatible solutes, I wish to compare results obtained in the current study for salt stress induction of *petite* colonies with results from other studies.

My results are different in some ways from Yanagashima's (1967). For instance, the results suggest that actively growing cells of *S. cerevisiae* are susceptible to various environmental changes resulting in varied proportions of *petite* mutants, whereas non-growing yeasts exposed to similar environment have a low proportion of *petite* mutants similar to that occuring spontaneously. Yanagashima (1967) reported a high incidence of respiratory deficient mutants in non-proliferating yeast. The diploid strain of *S. cerevisiae* var. *ellipsoideus* that he used may be more sensitive to salt

stress than our wild type diploid strain Y41. The degree of ploidy has previously been shown as a factor in acquired thermotolerance of *S. cerevisiae* (Wood 1956). This author found that diploid cells of *S. cerevisiae* are 2.5 times less sensitive to the lethal effects at 52°C than haploid strains. Hence it can be suggested that non-growing cells of haploid strain of *S. cerevisiae* var. *ellipsoideus* would have been resulted in a higher proportion of *petites* than encountered by the diploid ones, without considering the influence of a growing culture. The induction of *petite* mutation in some other yeast strains by salt solutions was not observed as clearly as in *S. cerevisiae* var. *ellipsoideus* (Yanagashima 1967).

Moreover, the proportion of *petite* mutants formed under the experimental conditions used in this study varies considerably among individual strains (see TABLE 3.4). It has been suggested that daughter cells are more likely to undergo spontaneous mutation than parental cell (James et al. 1977). Following pedigree analysis, it was suggested that either a selective distribution of defective mitochondria to the bud at cytokinesis, or retention by the mother cells of some factors that prevented the induction of defective mitochondria may be responsible. The high incidence of *petites* arising spontaneously in strain 182-6-3 (cdc 24) (see Tables 3.1 & 3.4) might be explained by the former mechanism. Since cdc 24 gene product is presumably intimately involved in bud emergence (Hartwell 1974; Hartwell et al. 1974), i.e. a mutation in this gene blocks budding; and the evidence that mtDNA recombination occurs extensively in yeast (see Evans 1982), it is likely that the high rate of spontaneous *petite* mutants in cdc 24 may be due to daughter cells missing some steps at the onset of budding, thus propagating *petite* cells.

Cultures of *S. cerevisiae* var. *ellipsoideus* were killed off more quickly in a medium containing sodium chloride than in potassium chloride (Yanagashima 1967) and, more respiratory deficient mutants arose from cultures containing sodium chloride than potassium chloride (see above). In addition, growth of *S. cerevisiae* var. *ellipsoideus* in cultures containing sodium with no added K^+ led to a substitution of intracellular K^+ by Na^+ (Conway and Moore 1954). This may be one of the reasons why *S. cerevisiae* var. *ellipsoideus* is more sensitive to Na^+ than to K^+, and, therefore, as a consequence leads to high incidence of *petites* . Let us return to discuss a possible role of compatible solute as a factor or one of the factors that may or may not suppress mutation.

Brown (1978) comments that, in addition to the compatible role of glycerol, its regulatory role in other areas such as physiology and biochemistry of yeasts should not be overlooked. He refers to the work of Wallis and Whittaker (1974) where they found that incubation in a simple solution of glycerol (2%) induces a high incidence of *petite* mutants. The genetic mechanism of this finding was not pursued but the authors speculated that glycerol caused a breakdown in the complex control system that yeasts required for maintaining mtDNA levels. As described above, glycerol stimulates both prokaryotic and eukaryotic transcription (Nakanishi et al. 1974; Bus and Stalter 1978). In addition, yeast alcohol dehydrogenase can be modified by glycerol at concentrations up to about 2.5 M (Myers and Jakoby 1975 cited in Brown 1978). This amount of glycerol decreased the Michaelis constants for this enzyme using ethanol and NAD^+, but increased the Km value for NADH. Moreover, the effect of glycerol in reducing the Michaelis constants of both ethanol and NAD^+ while increasing that for NADH, may lead to blocking acetaldehyde reduction, which, in turn, would favour glycerol production at the expense of ethanol. There are two cytosol dehydrogenases. One is constitutive (containing high Km ethanol value), which is responsible for reducing acetaldehyde to ethanol, whereas the other with a low Km (ethanol) can oxidize intracellular ethanol to acetaldehyde. *Petite* mutants produce only the former, while the normal colonies produced both (Wills 1976 cited in Brown 1978).

Yanagashima (1967) also emphasizes the importance of metabolic activity in explaining the mechanism of salt stress induced *petite* mutants. He studied the effect of nucleic acid base analogs such as 2-thiouracil and 8-azaguanine in response to cells incubated in a basal medium containing 0.45 M $SrCl_2$ and 2% glucose. It was observed that a concentration of 100 mg/litre of the nucleic acid analogs decreased the proportion of the mutants. He then concluded that nitrogen metabolism, particularly that related to RNA synthesis, is involved in the induction of *petites* by $SrCl_2$. In addition, Attfield (1987) suggested that the heatshock induction of trehalose in *S. cerevisiae* requires de novo RNA synthesis. With the above mentioned role of glycerol involvement in gene expression in *gal* RNA synthesis transcription system, it is reasonable to assume a possible involvement of compatible solutes in affecting the proportion of *petite* mutants generated under salt stress. The results obtained herein suggest that trehalose, as a compatible solute, and perhaps heat shock proteins, may also be responsible for suppressing *petite* mutagenesis.

4.4 CONCLUSIONS

(1) **An increase in the *petite* mutation rate** is observed for *S. cerevisiae* wild type (Y41), a temperature sensitive mutant (182-6-3, cdc 24), and a glycogen-deficient mutant (211-244-1A, *glc* 1) when subjected to **salt stress.** Lowering of temperature to 20°C did not reduce the mutation rate for the temperature sensitive strain.

(2) **An increase in the *petite* mutation rate** is observed for *Saccharomyces cerevisiae* wildtype strain Y41, and strain 211-244-1A, a mutant harboring *glc* 1 mutation, when subjected to **a temperature stress** at 52°C.

(3) *Petite* mutants from either wildtype strain Y41 or t.s. strain 182-6-3 (cdc 24) are **not** inherently **more resistant** to salt stress than the corresponding parental strains (*grandes* or normal colonies).

(4) Partial **protection** against *petite* mutagenesis induced at 52°C is observed following preconditioning in either 2% NaCl or at 45°C.

(5) Partial **protection** against cell death induced by salt stress and 52°C is observed following preconditioning in 2% NaCl or at 45°C for *S. cerevisiae* strains Y41 and 211-244-1A (*glc* 1). In addition, ***petite* mutagenesis** is not protected when 45°C stage two mid-exponential cultures were subjected to salt broth at 30°C.

(6) Incubation of stage two mid exponential cultures in salt broth media containing 10% NaCl that was already equilibrated at 52°C **reduces** the death rate and proportion of *petite* mutants.

(7) It is possible that **heat shock proteins** as well as **trehalose,** which increases during preconditioning at 45°C, may be sharing a role as **protector** against heat and salt stress-induced mutagenesis. In salt stress, the protecting effect of trehalose may only be effective in stationary phase.

The overall conclusion is that *petite* mutants that arose after transfer of mid-exponential phase to salt broth are not a result of **greater resistance (differential selection)** of the pre-existing *petite* strains to salt stress; but are attributable to a **mutagenic action** imposed by the salt stress.

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